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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
GARY L. CLAYMAN

Serial No.: 08/758,033

Filed: November 27, 1996

For: METHOD AND COMPOSITION FOR THE DIAGNOSIS AND TREATMENT OF CANCER

Group Art Unit: 1632

Examiner: K. Hauda

Atty. Dkt. No.: INRP:041/HYL

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BRIEF ON APPEAL

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BRIEF ON APPEAL

BOX AF
Hon. Asst. Commissioner of Patents
Washington DC 20231

Dear Sir:

This Brief is filed in response the final Office Action, mailed on April 12, 1999, and the Advisory Action, mailed on September 20, 1999, both regarding the above-captioned application. The fee for this brief is enclosed. No other fees are believed due in connection with this filing. However, should any other fees be due, or should appellant's check be missing, the Commissioner is authorized to debit Arnold, White & Durkee Deposit Account No. 1-2508/INRP:041/HYL.

I. Status of the Claims

Claims 1-25 were filed with the original application. Claims 26-145 have been added. Claims 15, 21-25, 78 and 79 were canceled in the first response, and claims 34, 35, 71, 72, 106, 107, 135, 136, 140, 141, 144 and 145 were canceled in the second response; however, it appears that these claims have not been canceled due to non-entry of the amendment (see below). In an accompanying amendment, claims 33-35, 69-72, 104-107, 133-136, 140, 141, 144 and 145 are canceled. Thus, claims 1-14, 16-20, 26-32, 36-68, 73-77, 80-103, 108-132, 137-139, 142 and 143 remain pending and are appealed.

II. Status of the Amendments

Amendments, offered in response to the final Office Action, were not entered. Appellant is filing an amendment concurrent with this brief, requesting cancellation of claims 33-35, 69-72, 104-107, 133-136, 140, 141, 144 and 145, and requesting the introduction of minor amendments to various of the claims.

III. Parties in Interest

The real parties in interest are the assignee, The Board of Regents, University of Texas System, the exclusive licensee, Introgen Therapeutics, and its sublicensee, Rhone Poulenc Rorer.

IV. Related Appeals and Interferences

There are no related appeals or interferences.

V. Summary of the Invention

The present invention deals with cancer gene therapy. More particularly, it addresses the use of adenoviral-p53 vectors for the treatment of various forms of cancer. Specification at page 3, lines 12-21. In a particular embodiment, the invention involves the use of a continuous perfusion protocol to treat a tumor site over a period of time. Specification at page 4, lines 8-11. In a second embodiment, the invention provides for treatment of microscopic residual disease, resulting from tumor resection. Specification at page 4, lines 2-4. In various dependent embodiments, the invention relates to treatment of both p53-mutated and p53-wild-type tumors. Specification at page 3, line 24.

VI. Issues on Appeal

A. Are claims 33-35, 69-72, 104-107, 133-136, 140, 141, 144 and 145 enabled?

B. Are claims 1-14, 16-20, 26-37, 74-77, 80-108, 140 and 144 indefinite?

C. Are claims 38-68, 73, 109-132 and 137 obvious over Liu *et al.*-1994 (Exhibit A) or Wills *et al.* (Exhibit B), in view of Zhang *et al.* (Exhibit C) or Bramwell (Exhibit D)?

D. Are claims 1-14, 16-20, 26-32, 36-68, 73-77, 80-132 and 137-145 obvious over Cajot *et al.* (Exhibit E), Katayose *et al.* (Exhibit F) or Srivastava *et al.* (Exhibit G), taken with Wills *et al.* or Liu *et al.*, in view of Zhang *et al.* or Bramwell?

VII. Grouping of the Claims

All of the pending claims will stand or fall separately and have been dealt with separately herein, in particular, Section IX.C.iv

VIII. Summary of the Argument

1. The rejections under 35 U.S.C. §112, first and second paragraphs, have been addressed by the minor amendments set forth in the accompanying amendments, and through cancellation of certain of the claims.

2. In connection with a first obviousness rejection, the examiner advances Lui-1994, Wills, Zhang and Brahmwell against claims that are directed to (a) post-operative treatment of microscopic residual disease (claims 38-68 and 73) and (b) continuous perfusion (claims 109-132 and 137). It is Appellant's position that none of the art in any way teaches or suggests the application of p53 gene therapy following surgical resection of a tumor as a means of treating microscopic residual disease.

Microscopic Residual Disease. The Wills reference says nothing about surgery, and Liu, at best, teaches away from the invention by teaching that the tumor should ***not*** be removed prior to p53 administration. All that Zhang teaches is that "some day" surgery "might" be used in combination with gene therapy – a reference so vague that it teaches nothing. Indeed, a reasonable interpretation of Zhang, when viewed in combination with Liu, is that it teaches away from the invention by suggesting that the surgery be performed as simply a means of exposing the primary tumor for intratumoral injection.

Continuous Perfusion. Appellant submits that the term "continuous perfusion" is well defined in the specification. The Examiner is obligated to consider the terms used in the claims in a manner that is consistent with the definition given those terms in the specification – it is indeed black letter patent law that the patent practitioner is his or her own lexicographer. Given the definition given the term "continuous perfusion" in the specification, as well as the common and ordinary meaning of this term in the art, it can in no way be equated with a single bolus injection as suggested by the Examiner. As such, the claims to "continuous perfusion" clearly distinguish over a single bolus injection.

3. In the second obviousness rejection, the Examiner rejects the "wild type p53 positive tumor therapy" claims (claims 1-14, 16-20, 26-32, 36-68, 73-77, 80-132 and 137-145) as obvious over Cajot, Katayose, Srivastava, Wills, Liu, Zhang and Brahmwell. With respect to Cajot *et al.*, these studies are unrelated to the actual therapy of human tumors, and are indeed fatally flawed for reasons detailed in depth in the response to final (to which the examiner offered no rebuttal). Second, the Srivastava and Katayose references evidence confusion in the art regarding actual therapy in humans, and are not even prior art against the present application. Third, and perhaps most convincingly, at the time of filing, the prior art evinced a state of utter confusion with regard to p53 treatment of p53-positive tumors. Again, the examiner has offered no counter-explanation of why any of this is not dispositive. From a factual standpoint, the rejection must fall.

IX. Argument

A. REJECTION UNDER 35 U.S.C. §112, FIRST PARAGRAPH

Claims 33-35, 69-72, 104-107, 133-136, 140, 141, 144 and 145 stand rejected as lacking enablement for combinations using any second gene other than p53. For claims 33-35, 69-72, 104-107, 133-136, it is the examiner's position that the specification fails to teach which genes will augment the anti-tumor effect of p53. Claims 140, 141, 144 and 145 are rejected as intravenous and oral administration are said to conflict with "direct" administration to a tumor.

Appellant traverses the rejection on the grounds that a) all of the recited genes are recognized to be useful in gene therapy applications, and methods are known in the art for their use, and b) there is no requirement under §112, first paragraph, that a second gene augment the action of p53. Nevertheless, since broader claims will encompass the use of p53 in the presence or absence of second genes, appellant has canceled the rejected dependent claims in order to reduce the number of issues on appeal. Similarly, though traversing the rejection, appellant has canceled claims 140, 141, 144 and 145, thereby reducing the issues on appeal.

B. REJECTION UNDER 35 U.S.C. §112, SECOND PARAGRAPH

Claims 1-14, 16-20, 26-37, 74-77, 80-108, 140 and 144 are rejected as indefinite under the second paragraph of §112. According to the examiner, it is unclear how intravenous administration, recited in claims 140 and 144, can be "direct" administration to a solid tumor. No grounds of rejection are given for claims 1-14, 16-20, 26-37, 74-77 and 80-108.

Appellant traverses the rejection of claims 140 and 144, but in an effort to reduce the number of issues on appeal, these claims have been canceled. Again, appellant reiterates that a response to the rejection of claims 1-14, 16-20, 26-37, 74-77 and 80-108 is not possible without an explanation of the rejection. Should the examiner advance any reasons for rejection, appellant request that it be deemed a new ground of rejection, and treated as such on appeal.

C. REJECTIONS UNDER 35 U.S.C. §103

i) Liu, Wills, Zhang and Brahmwell

Claims 38-68, 73, 109-132 and 137 are rejected as obvious over Liu *et al.*-1994 or Wills *et al.*, in view of Zhang *et al.* or Brahmwell. Liu and Wills are said to teach *in vivo* delivery and expression of p53 in tumors using an adenoviral expression system. It is the examiner's position that, though the references fail to teach treatment of microscopic residual disease or continuous perfusion, one of skill in the art would have recognized the benefit from performing the particular therapies. Thus, it is argued that the claims are obvious over the cited references. Appellant traverses.

(a) Tumor Resection Claims (38-68 and 73)

Appellant points out that none of the references relied upon by the Examiner in any way teach or suggest the treatment of microscopic residual tumors. Indeed, the references in no way teach or suggest administering the p53 gene to a tumor bed revealed by resection of all or a portion of the tumor. The Liu reference appears to teach only the administration of the p53 gene in adenovirus to SCCHN cell lines, or directly to the

primary tumor grown in skin flaps of nude mice. The only surgery taught by Liu concerns reopening of surgical "flaps" under which the primary tumors are grown. In Liu, the tumor is ***not*** removed prior to administration of the adeno-p53 and there is no suggestion that it be removed prior to administration of the adeno-p53.

The same can be said for Wills, which merely teaches the injection of adeno-p53 at the site of growing tumors in nude mice. Wills teaches ***nothing*** about combination with surgical therapy and says ***nothing*** about the resection of tumor masses prior to gene therapy.

If Appellant has misunderstood or overlooked some relevant teaching in either of these articles, the Examiner is requested to identify the teaching that is being relied upon.

The secondary references also fail to provide a basis for a conclusion of obviousness. The Examiner argues that Zhang suggests treating microscopic residual disease. However, all that Zhang teaches, and all that is apparently relied upon from that reference, is that surgery may at some point in time be used in combination with gene therapy (col. 2, p. 505). Appellant has been unable to identify any teaching relevant to the treatment of microscopic disease. The passage relied upon by the Examiner is so vague that it is virtually irrelevant and could be taken to mean almost anything or in fact nothing. For example, Zhang's reference to surgery could be taken as suggesting the injection the p53 gene into the primary tumor mass followed by resection of that same tumor mass (as opposed to tumor resection ***prior*** to p53 therapy as required by the claim). Alternatively, Zhang could merely be suggesting that surgery is a useful way of actually exposing the tumor itself for injection of the p53 gene. Neither of the foregoing possible

interpretations of Zhang would in any way obviate the treatment of microscopic residual tumors after tumor resection.

In fact, when taken in combination with Liu, the logical conclusion is that surgery is to be used merely to reveal an existing tumor mass rather than as a means of treating the primary tumor, followed by gene therapy of any residual microscopic growth. The reason for this is that the only type of "surgery" actually performed by Liu related to simply revealing the tumor followed by adeno-p53 administration directly to the tumor itself (see Liu, page 3663, "After 4 days, the animals were re-anesthetized, and the flaps were re-elevated for delivery [of the adeno-p53 to the tumor].") Thus, the combination of Liu with Zhang would in no way suggest removal of the tumor followed by treatment of the remaining resection bed.

More importantly, though, Zhang must be taken for its face value – and on its face, it says nothing about the treatment of microscopic residual disease. Zhang merely suggests that "someday" it may be possible to treat patients with combination gene therapy and surgery without providing any disclosure of details as to how this might "someday" be carried out.

Lastly, Bramwell is not at all relevant in that Appellant has been unable to identify any suggestion of combination therapy with surgery. If Appellant is in error, the Examiner is requested to point out that passage being relied upon. Barring such an eventuality, Appellant submits that Bramwell in fact teaches away from combination with surgery, in that it conspicuously fails to suggest such a possibility.

In conclusion, while it may be true that using surgery to enhance the therapeutic benefit of other therapies is known, this is a far cry from teaching or suggesting the

specific endeavor of treating, in post-operative fashion, microscopic residual disease that remains after tumor excision. In fact, Zhang does not teach treatment of microscopic residual disease, nor does it even allude to the problem of post-operative reoccurrence. Thus, it is submitted that the rejection is fatally defective in failing to provide a disclosure of an element of the claimed invention.

(b) Perfusion Claims (109-132 and 137)[1]

With regard to the "continuous perfusion" claims, Appellant respectfully traverses the Examiner's conclusion of obviousness. The Examiner appears to present two separate arguments in support of the rejection. First, the Examiner suggests that the phrase "continuous perfusion" could cover a single bolus injection. Secondly, the Examiner suggests, without support, that contacting the tumor with the virus for extended periods of time would be expected to be advantageous.

Performing the same analysis on the references, it is evident that no teaching of continuous perfusion can be found. Rather than fill the gap by attributing such knowledge to the skilled artisan, as the examiner attempts for treatment of post-operative sites, here the examiner simply construes "continuous perfusion" as broad enough to encompass a single bolus injection. This is both self-serving and wrong.

With respect to the definition of "continuous perfusion," Appellant directs the Examiner to page 33 of the specification. There, the following passage can be found:

> In certain embodiments, it may be desirable to provide a continuous supply of therapeutic composition to the patient. For intravenous or intraarterial

[1] Claims 56, 91, 112 and 120 address administration via "a catheter" and continuous perfusion for 1-2 hours. Though these claims are not rejected here, appellant incorporates the arguments under this heading in their argument for separate patentability of these claims.

> routes, this accomplished by drip system. For topical application, repeated application would be employed. For various approaches, delayed release formulations could be used that provided limited but constant amounts of therapeutic agent over [an] extended period of time. For internal application, continuous perfusion of the region of interest may be preferred. This could be accomplished by catheterization, post-operatively in some cases, followed by continuous administration of the therapeutic agent. The time period for perfusion would be selected by the clinician for the particular patient and situation, but times could range from about 1-2 hours, to 2-6 hours, to about 6-10 hours, to about 10-24 hours, to about 1-2 days, to about 1-2 weeks or longer.

Page 33, lines 19-27. This passage clearly defines what "continuous" means. It speaks in terms of time ***periods***, ranging upwards from about 1 hour. It speaks of drip systems, catheters, repeated topical applications, none of which can be twisted into support for the concept of a single injection. In short, this passage clearly refutes any possible interpretation of the claims to encompass single bolus injections.

Regarding the Examiner's comment that it was "well known in the art ... that the longer the vector is in contact with the cell, the greater the transduction efficiency," Appellant respectfully requests that such art being relied upon be made of record so that Appellant might respond in a meaningful fashion. If the Examiner is instead relying upon personal knowledge, the Examiner is respectfully requested to appropriately make such personal knowledge of record. See MPEP §2144.03.

It is respectfully submitted that the subject claims are now free of the prior art, and reconsideration is requested in light of the foregoing. Indeed, it is incumbent upon the examiner to come forward with specific reasons, based upon the prior art disclosures, as to why those of skill in the art would combine the cited references to arrive at the present invention ***at the time of the invention***. As discussed in the recent case of *In re Dembiczak*, Slip. Op. 98-1498 (April 28, 1999), strict adherence to this methodology will

prevent "fall[ing] victim to the insidious effect of a hindsight syndrome wherein that which only the inventor taught is used against its teacher." *Id.* at p. 7, citing *W.L. Gore & Assoc., Inc. v. Garlock, Inc.*, 220 USPQ 303, 313 (Fed. Cir. 1983). To the contrary, the examiner here has viewed ***the present invention*** first, and then assembled odd references which allegedly (though incompletely) disclose elements of the present invention. In so doing, the examiner has committed just the error warned against by *Dembiczak*, *Gore* and a host of other cases, namely, using hindsight as a foundation for the rejection. *Interconnect Planning Corp. v. Feil*, 227 USPQ 543, 547 (Fed. Cir. 1985) ("The invention must be view not with the blueprint drawn by the inventor, but in the state of the art that existed at the time.").

In both of the preceding rejections, the Examiner has simply asserted that the claimed invention is obvious. To the extent that the cited art can be said to disclose elements of the present invention, there has been no effort to establish that the references posit their own combination. However, a long line of cases establishes that the references must provide some basis for their use in conjunction with each other. "Broad conclusory statements regarding the teaching of multiple references, standing alone, are not 'evidence.'" *Dembiczak* at p. 9. Rather, the PTO "must identify specifically ... the reasons one of ordinary skill in the art would have been motivated to select the references and combine them." *In re Rouffet*, 47 USPQ2d, 1453, 1459 (Fed. Cir. 1998).

ii) Cajot, Katayose, Srivastava, Wills, Liu, Zhang and Brahmwell

Claims 1-14, 16-20, 26-32, 36-68, 73-77, 80-132 and 137-145 are next rejected under 35 U.S.C. §103 as obvious over Cajot, Katayose, or Srivastava, taken with Wills,

Liu, Zhang or Brahmwell. The primary references are said to teach that provision of p53 to p53-positive tumor cells results in inhibition of tumor cell growth. The secondary references are cited for various aspect of gene therapy. Together, the examiner indicates that these references obviate the treatment of p53-positive tumors *in vivo*. Appellant respectfully traverses.

Ignoring for the moment the issue of whether the secondary references support gene therapy generally, Appellant respectfully submits that the primary references do not, in fact, suggest to one of skill in the art the treatment of p53-positive tumor cells using p53 expression constructs. This is true for at least three reasons. First, as evidenced by the references themselves, there was a considerable difference of opinion in the field as to whether p53-positive cells were susceptible to treatment. Second, there also was some indication that *in vitro* studies, at least with respect to treatment of p53-positive tumors, were not predictive of *in vivo* efficacy. And third, at least one of the studies was fundamentally flawed, from a scientific standpoint, thereby reducing its probative value to a nullity. In light of these consideration, it is respectfully submitted, as explained in detail below, that no valid *prima facie* case could exist against the rejected claims.

(a) Katayose and Srivastava Evidence Confusion in the Field

As will be explained, Katayose and Srivastava fall far short of an endorsement of treating p53-positive tumors with a p53 expression construct. In fact, a more accurate portrayal of these teachings would be that there was considerable confusion in the field as to whether a p53 positive tumor ***in an actual human patient*** (as opposed to *in vitro* or in

a nude mouse) could be successfully treated. Indeed, this confusion can be seen as teaching away from treating a p53-positive tumor with additional exogenous p53.

- ***Katayose***

Turning first to Katayose, this study employed adeno-p53 constructs to examine the susceptibility of various tumor cell lines (p53 null, p53 mutant, p53 positive) to p53 gene therapy. As abstracted, the results indicated that "tumor cells that were null for p53 prior to infection ... and tumor cells that expressed mutant endogenous p53 protein ... were more sensitive to AdWTp53 cytotoxicity than cells that contained the wild-type p53 ...." This rather non-committal statement is clarified by the last line of the introduction which states that "these studies indicated that an adenovirus vector expressing wild-type p53 is markedly cytotoxic ***to tumor cells that have null or mutant p53 expression*** ..." (emphasis added). No mention is made of p53-positive cells. In addition, the last line of the abstract summarizes the authors conclusions: "These data suggest that endogenous p53 status is a determinant of AdWTp53-mediated cell killing of human tumors." The clear inference is that only p53 null or p53 mutant tumor cells are killed by AdWTp53, not tumor cells that are WTp53.

Katayose indeed actually teaches away from treating p53-positve tumor cells with p53 expression vector. In the Discussion on page 896, first column, second paragraph, it is stated that "There are several possible mechanisms by which high expression of wild-type p53 results in apoptosis in tumor cells devoid of p53 or expressing mutant p53, but not in tumor or normal cells expressing wild-type p53". Thus, Katayose is itself stating

quite clearly that expression of wild-type p53 would not be expected to effect apoptosis in a tumor which expresses wild-type p53.

The following additional comments also illuminate what the skilled artisan would take away from Katayose. "As shown in Fig. 3, *A* and *B*, infection of H-358 and MDA-MB-231 [p53 null and mutant, respectively] cells with AdWTp53 completely inhibited cell growth .... In contrast, MCF-7 cells [p53 positive] continued to proliferate although at a slower rate than control cells ...." Page 892, right hand column. "It appears that cells that express wild-type p53 were 5-250 times more resistant to the AdWTp53-mediated inhibitory effect on cell growth when compared with cells expressing no *p53* or mutant *p53*." Page 893, right hand column. "These results indicate that tumor cells null for *p53* or expressing an endogenous mutant *p53* undergo apoptosis following exposure to AdWTp53, whereas tumor cells or normal cells expressing wild-type p53 are resistant to apoptosis." Page 895, right hand column. "... [O]verexpression of wild-type p53 induced programmed cells death (apoptosis) of tumor cells devoid of wild-type p53 or expressing endogenous mutant *p53*, but not in tumor or normal cells expressing wild-type p53." Page 896, left hand column. ***These passages clearly indicate that the Katayose reference cannot be read as providing sufficient motivation for treating p53-positive cells. To the contrary, the reference suggests the opposite, that p53-positive cells are far less susceptible to such treatments***.

Srivastava

Srivastava also provides an insufficient basis for suggesting that one should ***clinically*** treat p53-positive cells with a p53 expression construct. In fact, the justification

of growth inhibitory effects by a hypothetical mutation in LNCaP p53 is demonstrative of the prior art teaching the opposite of that alleged by the Examiner. While the abstract states that "AdWTp53 vector exhibited a potent inhibitory effect on the growth of all [six] of human metastatic prostate cancer cells ...," the ensuing discussion paints a much muddier picture. For example, the authors state that:

> Since several previous studies did not observe cell growth inhibitory effects of exogenous p53 in tumor cells that already contained endogenous wt p53,[24,25] the inhibitory effects of AdWTp53 on LNCaP cells containing endogenous wt p53 was unexpected. However, in agreement with the previous observations,[18] we also did not detect a growth inhibitory effect of AdWTp53 on breast cancer cells, MCF7 containing endogenous wt p53 (data not shown).

Page 845, right hand column.

However, a later statement makes it clear that Srivastava believes that there must be another p53 function that is being supplied by the exogenous wt p53 in these cells: "However, it is possible that some as yet unknown function of p53 is defective in LNCaP cells..." see page 847, second column. This suggests that one skilled in the art was directed away from the idea of clinically treating p53 positive cells with a p53 expression construct. Otherwise, the results in LNCaP cells would not need the justification afforded by this statement. Thus, reading Srivastava, one of skill in the art would not be led to treat p53-positive cells with p53 gene therapy with any likelihood of success.

Taken together, Srivastava and Katayose present a very unclear view of whether the p53 status of a tumor cell is important in determining whether or not p53 gene therapy will be successful. In fact, a more likely interpretation, based on the data, is that p53-positive tumors are much less likely to respond to such therapy, and if they do respond, they do so far less than do other tumors.

(b) Extrapolating from In Vitro to In Vivo is Problematic at Best

According to the examiner, it would have been obvious, looking at the alleged p53-positive cell inhibition "successes" of Katayose, Srivastava and Cajot, to then move into the clinical realm according to the teachings of the secondary references. However, as the PTO is quick to tell appellant, extrapolation from *in vitro* data to *in vivo* efficacy is, at best, problematic. That position is, apparently, even true in this particular instance.

In Srivastava, it is noted that "[a] recent study has described an intriguing result in which an adenovirus-p53 expression vector did not inhibit the *in vitro* growth of a metastatic variant of LNCaP cells; however, the growth of these cells was inhibited *in vivo*.[31] This is evidence that there is at least some level of difficulty in predicting, at least in the context of treating p53-positive cells, whether *in vitro* results will hold in an *in vivo* environment. Additionally, the *in vitro* results referenced were contradictory to the data presented in Srivastava leaving doubt as to the success of *in vivo* p53 treatment of LNCap cells. Further evidence for the lack of a reasonable expectation of success is the fact that the investigators did in fact not report the treatment of LNCaP tumors in an *in vivo* mouse model, as implied in the final sentence on page 847 second column. As such, it is apparent that the examiner's straightforward extrapolation not only is not merited, but it is not supported by the evidence of record.

(c) Srivastava and Katayose Are Not Prior Art

In the previous response, appellant argued that Srivastava and Katayose were effectively removed as prior art by virtue of a) the January 1995 publications by Clayman

et al. and Liu *et al.*-1995, taken with b) appellant's previously filed declaration from Dr. Clayman (Exhibit H). According to the examiner, this declaration was a "Katz" type declaration and, hence, insufficient to antedate either of the references. Appellant respectfully traverses.

The prior Clayman declaration was submitted to identify the source of the "inventive" subject matter in the Clayman *et al.* and Liu *et al.*-1995 papers. From that declaration, it is clear that Dr. Clayman, the present inventor, was the source of that subject matter. Moreover, the publication of the subject matter of Clayman *et al.* and Liu *et al.*-1995 in January of 1995 indicates that, at the very least, this subject matter was in the hands of the present inventor prior to the publication of either Katayose or Srivastava.

In the interest of advancing the prosecution, however, Appellant provides a second declaration, also from Dr. Clayman (Exhibit I), which indicates that he had in his possession at least that much of the invention as did the references ***prior to their publication***. *In re Stempel*, 113 USPQ 77 (CCPA 1957). As such, appellant again respectfully submits that Srivastava and Katayose are not prior art against the present application. On this ground alone, the rejection should be withdrawn.

(d) The Prior Art Teaches Away/Failure of Others

There are a number of additional references that must be considered as teaching away from a conclusion of obviousness, and indeed further evidence the confusion in this field. These include the references of Baker *et al.* (*Science*, 249:912, 1990, Exhibit J) and Casey *et al.* (*Oncogene*, 6:1791, 1991, Exhibit K). Each of these references demonstrates

those *in vitro* attempts to achieve suppression of the neoplastic phenotype in p53-positive tumors ***failed*** to demonstrate suppression.

The examiner must consider the entirety of the prior art when assessing the obviousness issue, including evidence of failure of others and teaching away. The Baker and Casey references demonstrate failure in *in vitro* studies using p53 to suppress the growth of p53-positive cells. Such teachings must be taken as teaching away from pursuing the clinical application of p53 therapy in humans having wt p53 tumors. Furthermore, when these studies are further taken into consideration with the observation that *in vitro* results with p53 do not necessarily correlate with *in vivo* observations, the only reasonable conclusion is that there is no predictable success in the clinical application of p53 gene for the treatment of tumors expressing wild type p53.

(e) The Cajot Studies are Fatally Flawed

Despite what Cajot may or may not say, Appellant submits that the previously discussed confusion in the art regarding p53-positive cells and their treatment with p53 gene therapy is more than sufficient to rob the examiner's alleged *prima facie* case of the requisite likelihood of success. However, the Cajot paper suffers from an additional defect that should be noted on the record. It is submitted, respectfully, that this defect precludes reliance on Cajot for the simple reason that its results are invalid.

In order to understand more fully the problems with Cajot, it is first necessary to explain their studies in some detail. Human lung cancer cells ***in culture*** were transfected with vectors containing either wild-type or mutant p53 under the control of the CMV promoter and the neomycin resistance gene under the control of the SV40 promoter.

Cells were subjected to geneticin selection for 3-4 weeks. Thus, stable transformants were selected. The authors concluded that, because few colonies were observed with wild-type p53 expressing clones, there was an inhibitory effect on tumor cell growth by the p53 expressed in these cells.

What Cajot did not account for was the potent down-regulation of the SV40 promoter by p53. Subler *et al.* (1992); Jackson *et al.* (1993); Perrem *et al.* (1995) (Exhibits L, M and N). Of particular interest is the Subler *et al.* report, which also indicates the a mutation in p53 at position 143, which is the same as Cajot mutant p53, did not show this inhibitory effect.

Looking at the Cajot data, what does this all mean? Well, for one, the difference in the transfection efficiencies seen between wild-type and mutant p53 can be explained by the fact that wild-type p53 shuts down the SV40, eliminating neomycin expression, and thereby eliminating cells because of geneticin toxicity, ***not because of tumor inhibition***. The skilled artisan, being aware of both Subler *et al.* and Cajot, would therefore have dismissed Cajot's observations as invalid.

Even Cajot bears out the notion that the remaining tumor clones survived because the p53 gene being expressed became mutated, thereby avoiding the SV40 down-regulation of neomycin: "In contrast, no normal-size transcript characteristic of exogenous p53 was detected in any of the wild-type p53 clones analyzed." Page 6957, right hand column. Further, they state that "[n]o increase in the expression level of this M_r 53,000 band was observed in the wild-type p53 clones, which correlates with the absence of normal-size exogenous transcript detected by Northern analysis." Page 6957, right hand column. Also, in Fig. 3, it should be noted that the tumorigenicity studies

were conducted with a clone that admittedly does not express a wild-type p53 product, as the transcript is larger than normal (1.8 kB), and the protein is smaller (45 kD), than true wild-type p53. Thus, based on a singular experiment using an admittedly mutant p53, there is no valid conclusion that can be drawn from the study regarding the effect of ***wild-type*** p53 on tumorigenicity in p53-positive tumor cells.

(f) Current Clinical Data Supports the Present Claims

The current clinical data supports a conclusion of surprising and unexpected results in the context of the clinical application of the present invention. The clinical data further provides support for the conclusion that when applied in a clinical context, the invention is applicable in the treatment of wt p53 expressing tumors virtually to the same degree as in the context of non-wt p53 expressing tumors. This is most surely a surprising and unexpected result.

In the Phase I study reported in Clayman *et al., J. Clin. Oncol.* 16:221-2232 (1998) (Exhibit O), thirty-three patients with recurrent head & neck cancer were treated with intratumoral injections of Ad-p53. The treatment regimen consisted of at least one course of Ad-p53 (three times a week for two weeks). Of these, eighteen patients had non-resectable tumors (and received multiple treatment courses with two week rest between courses; continuing until disease progression or withdrawal of consent), permitting post-treatment assessment of tumor progression. Of these eighteen, twelve were p53+ by sequencing of tumor cell DNA. Of these twelve, two patients had greater than 50% tumor regression, four had stable disease, five had progressive disease, and in one the outcome of treatment could not be evaluated. By comparison, of the remaining

six non-resectable patients, one was non-evaluable for p53 status. Of the five patients with mutated p53 genes, four exhibited progressive disease while two exhibited stable disease. Of the 33 patients entered in the study, the remaining 15 underwent complete resection of their tumor three days after a single course of treatment, and could not be rigorously assessed for clinical response.

Similarly, phase II Clinical data has recently become available for the treatment of head & neck cancer using Ad-p53. As discussed in the accompanying declaration of Dr. James A. Merritt (Exhibit P), tumors of various p53 status (as determined by sequencing of exons 1-10) have been treated with Ad-p53. These Phase I and II clinical data demonstrate that p53+ tumors were susceptible to treatment by Ad-p53. A summary of the data are show below:

SUMMARY OF PHASE II CLINICAL DATA BY p53 STATUS

p53 status*	CR	PR	SD	PD
p53+	0	2	6	21
p53-	0	1	6	27

* - as determined by sequencing of exons 1-10
CR – complete response; PR – partial response; SD – stable disease; PD – progressive disease

Scoring a complete response as 3, a partial response as 2, stable disease as 1, and progressive disease as 0, patients with p53-positive tumors had an efficacy rating of 0.42,

as compared to 0.29 for those having p53- tumors. Using this analysis, Ad-p53 was even ***more*** effective at treating p53+ tumors than p53- tumors.

This result could not have been predicted from the cited art. Thus, it again is respectfully submitted that those of skill in the art would not have found the present invention obvious

iii) Obviousness Requires Both a Teaching and a Suggestion ***In the Art***

As set out above, the examiner has advanced rejections of claims drawn to post-operative treatment of residual disease and continuous perfusion without any clear indication, in the cited art, that the skilled artisan should make these modifications. It is Appellant's position, therefore, that the examiner has failed to set forth a *prima facie* case of obviousness.

The Federal Circuit has held that a reference or references must provide, in order to support an obviousness rejection, a) detailed enabling methodology for practicing the claimed invention, b) a suggestion for modifying the claimed invention, and c) evidence suggesting that the invention would be successful if made. *In re O'Farrell*, 7 USPQ2d 1673 (Fed. Cir. 1988).

What is explicit (though often overlooked) in this statement is that the examiner must find these three elements ***in the cited art***, not in appellant's disclosure. *In re Soli*, 137 USPQ 797 (CCPA 1963) ("When, as in the instant case, the Patent Office finds, in the words of 35 USC §103, 'differences between the subject matter sought to be patented and the prior art,' it may not, without some basis in logic or scientific principle, merely alleged that such differences are either obvious or of no patentable significance and

thereby force an [applicant] to prove conclusively that it is wrong.") 137 USPQ at 801.

Here, the examiner has not been able to point to any disclosure in the cited references that suggest modification of the art to arrive at the claimed invention. As such, the rejection appears based on hindsight, not any teachings in the cited references. This, however, is forbidden under an obviousness analysis by the relevant case law. *In re Carroll*, 202 USPQ 571 (CCPA 1979) ("One of the more difficult aspects of resolving questions of non-obviousness is the necessity 'to guard against slipping into the use of hindsight.'"), citing *Graham v. John Deere Co.*, 148 USPQ 459 (U.S. Sup. Ct. 1965).

iv) The Non-Obviousness of Specific Claims

The arguments set forth above is intended to go the non-obviousness of all of the pending claims. Appellant would now address the additional non-obviousness considerations presented by the various dependant claims, which do not stand or fall together. Appellant will consider the non-obviousness of each dependant claim that is being argued as separately patentable as required by the rules.

Claims 2, 39, 76 and 111

Claims 2, 39 and 76 are directed to inhibiting the growth of a carcinoma, glioma, sarcoma or melanoma. These tumor types are submitted to be even further removed from the cells of the Cajot reference, which dealt with lung cancer cell lines growing in culture. As discussed above, Cajot is believed to the only prior art reference considered which in any way concerned p53-positive cells, albeit lung cancer cells *in vitro* and not an actual tumor in a human patient. It is respectfully submitted that a teaching with

respect to lung cancer cell lines is in no way predictive of treatment of a "solid tumor" comprised of actual carcinoma, glioma, sarcoma or melanoma tumors in a patient.

Claims 10 and 121

Claims 10 and 121 are further removed from Cajot as well, in light of the fact that the only animal studies set forth in Cajot are studies involving nude mice. The PTO has consistently taken the position that studies in nude mice, which have no immune system, are inherently unreliable as a predictor of efficacy and utility in humans. Thus, a claim specifically to humans – which are supported by actual human studies – must be accepted as surprising and unexpected vis-à-vis prior art relating only to nude mice.

Claim 12

Claim 12 is directed to at least two administrations of the p53 therapeutic agent to a subject having a p53-positive tumor, followed by tumor resection, and followed by an additional administration of the p53 therapeutic agent. There is simply no prior art that in any way addresses this claim, and certainly no *prima facie* case has even been attempted with respect to this claim.

Claim 17

Claim 17 is directed to continuous perfusion of a natural or artificial body cavity. This is a very particular mode of delivery, and addresses the issue of both tumor type (in

a natural body cavity) and post-resection treatment (artificial body cavity). The prior art is totally silent on this aspect of the invention.

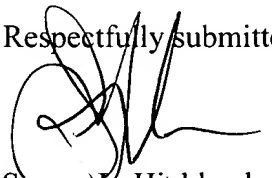
Claims 28, 29, 31, 61, 62, 63, 65, 67, 96, 100, 102, 125, 126, 127, 128, 129 and 131

Claim 28, 29, 31, 61, 62, 63, 65, 67, 96, 100, 102, 125, 126, 127, 128, 129 and 131 all claim various aspect of combination therapies with DNA damaging agents, including both radio- and chemotherapies, administration of the DNA damaging agent before, after, or before and after resection. The cited art does not address the use of DNA damaging agents, much less their combination with tumor resection, treatment of microscopic residual disease, or treatment of p53-positive cancers.

X. Conclusion

In light of the preceding, appellant respectfully submits that all of the remaining claims are enabled, definite and non-obvious. Therefore, it is respectfully requested that the Board reverse all grounds for rejection.

Respectfully submitted,



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Date: 11/9/99

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• see p. 84 spec. (clinical data) Phase I trials

red circle separately argued

• decl. Dr Merritt ... Phase II trials

APPENDIX 1: PENDING CLAIMS

1. (Three times amended) A method of inhibiting growth of a p53-positive tumor cell in a [mammlian] mammalian subject with a solid tumor comprising the steps of:

   (a) providing a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter; and

   (b) directly administering said viral expression construct to said tumor *in vivo*, the administration resulting in expression of said functional p53 polypeptide in cells of said tumor and inhibition of tumor cell growth,

   wherein said tumor comprises cells that express a functional p53 polypeptide.

2. The method of claim 1, wherein said tumor is selected from the group consisting of a carcinoma, a glioma, a sarcoma, and a melanoma.

3. The method of claim 1, wherein said tumor cell is malignant.

4. The method of claim 1, wherein said tumor cell is benign.

5. The method of claim 1, wherein said tumor is a tumor of the lung, skin, prostate, liver, testes, bone, brain, colon, pancreas, head and neck, stomach, ovary, breast or bladder.

6. The method of claim 1, wherein said viral expression construct is selected from the group consisting of a retroviral vector, an adenoviral vector and an adeno-associated viral vector.

7. The method of claim 6, wherein said viral vector is a replication-deficient adenoviral vector.

8. The method of claim 7, wherein said replication-deficient adenoviral vector is lacking at least a portion of the E1-region.

9. The method of claim 8, wherein said promoter is a CMV IE promoter.

10. The method of claim 1, wherein said subject is a human.

11. The method of claim 7, wherein the expression vector is administered to said tumor at least a second time.

12. The method of claim 11, wherein said tumor is resected following at least a second administration, and an additional administration is effected subsequent to said resection.

13. The method of claim 1, wherein said expression vector is administered in a volume of about 3 ml. to about 10 ml.

14. (Amended) The method of claim 11, wherein the amount of adenovirus [administered] in each [contacting] administration is between about 10^7 and 10^{12} pfu.

16. The method of claim 1, wherein the expression construct is injected into a natural or artificial body cavity.

17. The method of claim 16, wherein said injection comprises continuous perfusion of said natural or artificial body cavity.

18. The method of claim 16, wherein said contacting is via injection into an artificial body cavity resulting from tumor excision.

19. The method of claim 1, wherein the p53-encoding polynucleotide is tagged so that expression of p53 from said expression vector can be detected.

20. The method of claim 19, wherein the tag is a continuous epitope.

26. The method of claim 1, wherein said tumor is contacted with said expression construct at least twice.

27. The method of claim 26, wherein said multiple injections comprise about 0.1-0.5 ml volumes spaced about 1 cm apart.

28. The method of claim 1, further comprising contacting said tumor with a DNA damaging agent.

29. The method of claim 28, wherein said DNA damaging agent is a radiotherapeutic agent.

30. The method of claim 29, wherein said radiotherapeutic agent is selected from the group consisting of γ-irradiation, x-irradiation, uv-irradiation and microwaves.

31. The method of claim 28, wherein said DNA damaging agent is a chemotherapeutic agent.

32. The method of claim 31, wherein said chemotherapeutic agent is selected from the group consisting of adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, verapamil, doxorubicin, podophyllotoxin and cisplatin.

33. (Canceled) The method of claim 1, further comprising contacting said tumor with a cytokine.

34. (Canceled) The method of claim 1, further comprising contacting said tumor with a second therapeutic gene other than a gene encoding a *p53* polypeptide.

35. (Canceled) The method of claim 34, wherein said second therapeutic gene is selected from the group consisting of a Dp gene, p21, p16, p27, E_2F, Rb, APC, DC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, *ras*, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl*, *abl*, Bax, Bcl-X_s and E1A.

36. The method of claim 1, wherein said tumor is located into a body cavity selected from the group consisting of the mouth, pharynx, esophagus, larynx, trachea, pleural cavity, peritoneal cavity, bladder interior and colon lumen.

37. The method of claim 11, wherein said tumor is contacted with said expression construct at least six times within a two week treatment regimen.

38. A method for inhibiting microscopic residual tumor cell growth in a mammalian subject comprising the steps of:

(a) identifying a mammalian subject having a resectable tumor:
(b) resecting said tumor; and
(c) administering to a tumor bed revealed by resection a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter, the administration resulting in expression of said functional p53 polypeptide in said tumor cells and inhibition of their growth.

39. The method of claim 38, wherein said resectable tumor is a squamous cell carcinoma.

40. The method of claim 38, wherein the endogenous p53 of said resectable tumor is mutated.

41. The method of claim 38, wherein the endogenous p53 of said resectable tumor is wild-type.

42. The method of claim 38, wherein said tumor is a tumor of the lung, skin, prostate, liver, testes, bone, brain, colon, pancreas, head and neck, stomach, ovary, breast or bladder.

43. The method of claim 38, wherein said viral expression construct is selected from the group consisting of a retroviral vector, an adenoviral vector and an adeno-associated viral vector.

44. The method of claim 43, wherein said adenoviral vector is a replication-deficient adenoviral vector.

45. The method of claim 44, wherein said replication-deficient adenoviral vector is lacking at least a portion of the E1-region.

46. The method of claim 38, wherein said promoter is a CMV IE promoter.

47. The method of claim 38, wherein the resulting tumor bed is contacted with said expression construct at least twice.

48. The method of claim 38, wherein said expression construct is contacted with said tumor bed prior to closing of the incision.

49. The method of claim 44, wherein said the tumor bed is contacted with from about 10^6 to about 10^9 infectious adenoviral particles.

50. The method of claim 47, further comprising contacting said tumor with said expression construct prior to resecting said tumor.

51. The method of claim 50, wherein said tumor is injected with said expression construct.

52. The method of claim 51, wherein said tumor is injected with about 10^6 to about 10^9 infectious adenoviral particles.

53. The method of claim 51, wherein said tumor is injected with a total of about 1 ml to about 10 ml.

54. The method of claim 51, wherein said tumor is injected at least twice.

55. The method of claim 54, wherein each of said injections comprise about 0.1 ml to about 0.5 ml volumes spaced about 1 cm apart.

56. The method of claim 38, wherein the resulting tumor bed is contacted with said expression construct through a catheter.

57. The method of claim 54, wherein said contacting comprises about 10^6 to about 10^9 infectious adenoviral particles.

58. The method of claim 54, wherein said expression construct is contacted with said tumor in total of about 3 ml to about 10 ml.

59. The method of claim 38, wherein the *p53* polynucleotide is tagged so that expression of a p53 polypeptide can be detected.

60. The method of claim 59, wherein the tag is a continuous epitope.

61. The method of claim 38, further comprising contacting said tumor with a DNA damaging agent.

62. The method of claim 61, wherein said DNA damaging agent is contacted before resection.

63. The method of claim 61, wherein said DNA damaging agent is contacted after resection.

64. (Amended) The method of claim 61, wherein said DNA damaging agent is contacted [contacting] before and after resection.

65. The method of claim 61, wherein said DNA damaging agent is a radiotherapeutic agent.

66. The method of claim 65, wherein said radiotherapeutic agent is selected from the group consisting of γ-irradiation, x-irradiation, uv-irradiation and microwaves.

67. The method of claim 61, wherein said DNA damaging agent is a chemotherapeutic agent.

68. The method of claim 67, wherein said chemotherapeutic agent is selected from the group consisting of adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, verapamil, doxorubicin, podophyllotoxin and cisplatin.

69. (Canceled) The method of claim 38, further comprising contacting said tumor with a cytokine.

70. (Canceled) The method of claim 69, wherein said cytokine is selected from the group consisting of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, TGF-β, GM-CSF, M-CSF, TNFα, TNFβ, LAF, TCGF, BCGF, TRF, BAF, BDG, MP, LIF, OSM, TMF, PDGF, IFN-α, IFN-β and IFN-γ.

71. (Canceled) The method of claim 38, further comprising contacting said tumor with a second therapeutic gene other than a gene encoding a p53 polypeptide.

72. (Canceled) The method of claim 71, wherein said second therapeutic gene is selected from the group consisting of a Dp gene, p21, p16, p27, E_2F, Rb, APC, DC,

NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, *ras*, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl*, *abl*, Bax, Bcl-X_S and E1A.

73. The method of claim 38, wherein said tumor is located into a body cavity selected from the group consisting of the mouth, pharynx, esophagus, larynx, trachea, pleural cavity, peritoneal cavity, bladder interior and colon lumen.

74. A method for inhibiting growth of a p53-positive tumor cell in a mammalian subject having a solid tumor comprising the steps of:

(a) surgically revealing said tumor; and
(b) directly administering to said tumor a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter, the administration resulting in expression of said functional p53 polypeptide in said tumor cells and inhibition of their growth.

75. The method of claim 74, wherein said tumor is malignant.

76. The method of claim 74, wherein said tumor is a squamous cell carcinoma.

77. The method of claim 74, wherein said tumor is benign.

80. The method of claim 74, wherein said tumor is a tumor of the lung, skin, prostate, liver, testes, bone, brain, colon, pancreas, head and neck, stomach, ovary, breast or bladder.

81. The method of claim 74, wherein said viral expression construct is selected from the group consisting of a retroviral vector, an adenoviral vector and an adeno-associated viral vector.

82. The method of claim 81, wherein said adenoviral vector is a replication-deficient adenoviral vector.

83. The method of claim 82, wherein said replication-deficient adenoviral vector is lacking at least a portion of the E1-region.

84. The method of claim 74, wherein said promoter is a CMV IE promoter.

85. The method of claim 74, wherein said tumor is contacted with said expression construct at least twice.

86. The method of claim 74, wherein said expression construct is contacted with said tumor prior to close of the incision.

87. The method of claim 82, wherein said tumor is contacted with from about 10^6 to about 10^9 infectious adenoviral particles.

88. The method of claim 74, wherein said tumor is contacted with said expression construct in a total of about 1 ml to about 10 ml.

89. The method of claim 74, wherein said tumor is injected at least twice.

90. The method of claim 89, wherein each of said injections comprise about 0.1 ml to about 0.5 ml volumes spaced about 1 cm apart.

91. The method of claim 74, wherein said tumor is contacted with said expression construct through a catheter.

92. The method of claim 91, wherein said tumor is contacted with about 10^6 to about 10^9 infectious adenoviral particles.

93. The method of claim 91, wherein said tumor is contacted with an expression construct in a total of about 3 ml to about 10 ml.

94. The method of claim 74, wherein the p53 polynucleotide is tagged so that expression of a *p53* polypeptide can be detected.

95. The method of claim 94, wherein the tag is a continuous epitope.

96. The method of claim 74, further comprising contacting said tumor with a DNA damaging agent.

97. The method of claim 96, wherein said DNA damaging agent is contacted with said tumor before resection.

98. The method of claim 96, wherein said DNA damaging agent is contacted with said tumor after resection.

99. The method of claim 96, wherein DNA damaging agent is contacted with said tumor before and after resection.

100. The method of claim 96, wherein said DNA damaging agent is a radiotherapeutic agent.

101. The method of claim 100, wherein said radiotherapeutic agent is selected from the group consisting of γ-irradiation, x-irradiation, uv-irradiation and microwaves.

102. The method of claim 96, wherein said DNA damaging agent is a chemotherapeutic agent.

103. The method of claim 102, wherein said chemotherapeutic agent is selected from the group consisting of adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, verapamil, doxorubicin, podophyllotoxin and cisplatin.

104. (Canceled) The method of claim 74, further comprising contacting said tumor with a cytokine.

105. (Canceled) The method of claim 104, wherein said cytokine is selected from the group consisting of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, TGF-β, GM-CSF, M-CSF, TNFα, TNFβ, LAF, TCGF, BCGF, TRF, BAF, BDG, MP, LIF, OSM, TMF, PDGF, IFN-α, IFN-β, and IFN-γ.

106. (Canceled) The method of claim 74, further comprising contacting said tumor with a second therapeutic gene other than a gene encoding a p53 polypeptide.

107. (Canceled) The method of claim 106, wherein said second therapeutic gene is selected from the group consisting of a Dp gene, p21, p16, p27, E_2F, Rb, APC, DC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, *ras*, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl*, *abl*, Bax, Bcl-X_S and E1A.

108. The method of claim 74, wherein said tumor is located in a body cavity selected from the group consisting of the mouth, pharynx, esophagus, larynx, trachea, pleural cavity, peritoneal cavity, bladder interior and colon lumen.

109. (Twice amended) A method of inhibiting tumor cell growth in a mammalian subject having a solid tumor comprising the step of continuously perfusing a tumor site in said patient with a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter, the administration resulting in expression of said functional p53 polypeptide in cells of said tumor and inhibition of their growth.

110. The method of claim 109, wherein said tumor is malignant.

111. The method of claim 109, wherein said tumor is a squamous cell carcinoma.

112. The method of claim 109, wherein said tumor is benign.

113. The method of claim 109, wherein the endogenous p53 of said tumor is mutated.

114. The method of claim 109, wherein the endogenous p53 of said tumor is wild-type.

115. The method of claim 109, wherein said tumor is a tumor of the lung, skin, prostate, liver, testes, bone, brain, colon, pancreas, head and neck, stomach, ovary, breast or bladder.

116. The method of claim 116, wherein said viral expression construct is selected from the group consisting of a retroviral vector, an adenoviral vector and an adeno-associated viral vector.

117. The method of claim 116, wherein said adenoviral vector is a replication-deficient adenoviral vector.

118. The method of claim 117, wherein said replication-deficient adenoviral vector is lacking at least a portion of the E1-region.

119. The method of claim 109, wherein said promoter is a CMV IE promoter.

120. The method of claim 109, wherein said tumor site is perfused from about one to two hours.

121. The method of claim 109, wherein said subject is a human.

122. The method of claim 109, wherein said tumor site is contacted with said expression vector through a catheter.

123. The method of claim 109, wherein the p53 polynucleotide is tagged so that expression of a *p53* polypeptide can be detected.

124. The method of claim 123, wherein the tag is a continuous epitope.

125. The method of claim 109, further comprising contacting said tumor with a DNA damaging agent.

126. The method of claim 125, wherein said tumor site is contacted with said DNA damaging agent before resection.

127. The method of claim 125, wherein said tumor site is contacted with said DNA damaging agent after resection.

128. The method of claim 125, wherein said tumor site is contacted with said DNA damaging agent before and after resection.

129. The method of claim 125, wherein said DNA damaging agent is a radiotherapeutic agent.

130. The method of claim 129, wherein said radiotherapeutic agent is selected from the group consisting of γ-irradiation, x-irradiation, uv-irradiation and microwaves.

131. The method of claim 125, wherein said DNA damaging agent is a chemotherapeutic agent.

132. The method of claim 131, wherein said chemotherapeutic agent is selected from the group consisting of adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, verapamil, doxorubicin, podophyllotoxin and cisplatin.

133. (Canceled) The method of claim 109, further comprising contacting said tumor with a cytokine.

134. (Canceled) The method of claim 133, wherein said cytokine is selected from the group consisting of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, TGF-β, GM-CSF, M-CSF, TNFα, TNFβ, LAF, TCGF, BCGF, TRF, BAF, BDG, MP, LIF, OSM, TMF, PDGF, IFN-α, IFN-β, and IFN-γ.

135. (Canceled) The method of claim 74, further comprising contacting said tumor with a second therapeutic gene other than a gene encoding a p53 polypeptide.

136. (Canceled) The method of claim 135, wherein said second therapeutic gene is selected from the group consisting of a Dp gene, p21, p16, p27, E_2F, Rb, APC, DC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, *ras*, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl*, *abl*, Bax, Bcl-X_s and E1A.

137. The method of claim 109, wherein said tumor is located into a body cavity selected from the group consisting of the mouth, pharynx, esophagus, larynx, trachea, pleural cavity, peritoneal cavity, bladder interior and colon lumen.

138. The method of claim 1, wherein said expression vector is administered topically.

139. The method of claim 1, wherein said expression vector is administered intratumorally.

140. (Canceled) The method of claim 1, wherein said expression vector is administered intravenously.

141. (Canceled) The method of claim 1, wherein said expression vector is administered orally.

142. The method of claim 74, wherein said expression vector is administered topically.

143. The method of claim 74, wherein said expression vector is administered intratumorally.

144. (Canceled) The method of claim 74, wherein said expression vector is administered intravenously.

145. (Canceled) The method of claim 74, wherein said expression vector is administered orally.

[CANCER RESEARCH 54, 3662–3667, July 15, 1994]

Advances in Brief

Growth Suppression of Human Head and Neck Cancer Cells by the Introduction of a Wild-Type *p53* Gene via a Recombinant Adenovirus[1]

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Abstract

Mutations of the *p53* gene constitute one of the most frequent genetic alterations in squamous cell carcinoma of the head and neck (SCCHN). In this study, we introduced wild-type *p53* into two separate SCCHN cell lines via a recombinant adenoviral vector, Ad5CMV-p53. Northern blotting showed that following infection by the wild-type *p53* adenovirus (Ad5CMV-p53), cells produced up to 10-fold higher levels of exogenous *p53* mRNA than cells treated with vector only (without *p53*). Western blotting showed that the increased levels of p53 protein produced in the Ad5CMV-p53-infected cells were a reflection of *p53* mRNA expression. *In vitro* growth assays revealed growth arrest following Ad5CMV-p53 infection as well as cell morphological changes consistent with apoptosis. *In vivo* studies in nude mice with established s.c. squamous carcinoma nodules showed that tumor volumes were significantly reduced in mice that received peritumoral infiltration of Ad5CMV-p53. These data suggest that Ad5CMV-p53 may be further developed as a potential novel therapeutic agent for SCCHN since introduction of wild-type p53 into SCCHN cell lines attenuates their replication and tumor growth.

Introduction

Patients with SCCHN[3] are afflicted with a disease process that often has profound effects on speech, swallowing, and cosmesis. Furthermore, the overall rate of survival among these patients has remained unchanged at approximately 45% for nearly 30 years since contemporary surgery and radiation therapy were instituted (1). Treatment failures among these patients remain local and regional; only 10–15% of patients with the disease die of distant metastasis alone (2).

Although we have gained in understanding of the molecular events in the initiation and progression of SCCHN, they continue to require intensive investigation. A recent study identifying loss of heterozygosity of chromosome 9p21–22 as the most frequent genetic alteration in SCCHN suggested that this may be an early event in progression toward this neoplasm (3). Additionally, amplification and/or overexpression of cellular and nuclear oncogenes, such as c-*erB*-1 (4), *int*-2 (5), *bcl*-1 (6) and c-*myc* (7), have been documented in these cancers. The tumor suppressor gene *p53* has been the subject of immense interest and investigation in recent years. Alterations in the *p53* gene, including deletion, insertion, and point mutation, are the most frequent genetic events in many different carcinomas, such as those of the colon (8), breast (9), and lung (10), as well as soft-tissue sarcomas and leukemias (11). Several investigators have demonstrated the high frequency of *p53* gene alterations in SCCHN (12, 13).

There is considerable evidence implicating mutations of the *p53* gene in the etiology of many human cancers (14). Reports have demonstrated that growth of several different human cancer cell lines, including representatives of colon cancer (15), glioblastoma (16), breast cancer (17), and osteosarcoma (18), can be functionally suppressed by DNA transfection or retrovirus-mediated transfer of the wild-type *p53* gene. This gene may have an important role not only in cell growth but in apoptosis (programmed cell death). Induction of exogenous expression of wild-type *p53* has been shown to induce apoptosis in colon cancer cell lines (19) and in human lung cancer spheroids (20).

The adenoviral vector has emerged as a leading candidate for *in vivo* gene therapy in the past few years. It enjoys an advantage over traditional DNA transfection and retroviral transfer in its high efficiency of transferring potentially therapeutic genes into a wide range of host cells (21). The recently created adenoviral vector contains wild-type *p53* (Ad5CMV-p53; Ref. 22) provides us with an attractive delivery system to investigate the effect of exogenous wild-type *p53* on SCCHN cell lines both *in vitro* and *in vivo*. The outcome of this study indicates that further development of the p53 adenovirus other novel molecular therapies for SCCHN is warranted.

Materials and Methods

Cell Lines and Culture Conditions. Human SCCHN cell lines and Tu-177 were both established at the Department of Head and Surgery, M. D. Anderson Cancer Center. Tu-138 and Tu-177 were established from a gingivo-labial moderately differentiated squamous carcinoma and a poorly differentiated squamous carcinoma of the larynx, respectively. These lines were developed via primary explant technique and are cytokeratin positive and tumorigenic in athymic nude and SCID mice. These cells were grown in DMEM/F12 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin/streptomycin.

Recombinant Adenovirus Preparation and Infection. The recombinant p53 adenovirus (Ad5CMV-p53; Ref. 22) contains the CMV promoter, wild-type p53 cDNA, and SV40 polyadenylation signal in a minigene inserted into the E1-deleted region of modified Ad5. Viral stocks were propagated in 293 cells. Cells were harvested 36–40 h after infection, resuspended in phosphate-buffered saline, and lysed; cell debris removed by subjecting the cells to CsCl gradient purification. Concentrated virus was dialyzed, aliquoted, and stored at −80°C. Infection was carried out by addition of the virus to the DMEM/F12 medium and 2% FBS to the monolayers. The cells were incubated at 37°C for 60 min with occasional agitation. Then complete medium (DMEM/F12–10% FBS) was added and cells were incubated at 37°C for the desired length of time.

Northern Blot Analysis. Total RNA was isolated by the guanidium thiocyanate method of Chomczynski and Sacchi (23). Northern blot hyb performed on 20 μg of total RNA. The membrane was hyb

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[3] The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; DMEM/F12, Dulbecco's modified Eagle's medium/Ham's F-12 medium; FBS, fetal bovine serum; Ad5, adenovirus 5; CMV, cytomegalovirus; Ad5CMV-p53, wild-type p53 adenovirus; cDNA, complementary DNA; MOI, multiplicity of infection; SDS, sodium dodecyl sulfate; β-gal, β-galactosidase; dl312, replication-defective adenovirus; PFU, plaque forming units.

cDNA probe labeled by the random primer method in 5 × SSC-5 × Denhardt's solution-0.5% SDS-denatured salmon sperm DNA (20 μg/ml). The membrane was also stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase cDNA for RNA loading control. The relative quantities of *p53* expressed were determined by densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

Western Blot Analysis. Total cell lysates were prepared by sonicating the cells 24-h postinfection in RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) for 5 s. Fifty μg of protein from samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond-ECL membrane (Amersham). The membrane was blocked with Blotto/Tween (5% nonfat dry milk, 0.2% Tween 20, and 0.02% sodium azide in phosphate-buffered saline) and probed with the primary antibodies, mouse anti-human p53 monoclonal antibody PAb1801 and mouse anti-human β-actin monoclonal antibody (Amersham), and the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN). The membrane was processed and developed as the manufacturer suggested.

Immunohistochemical Analysis. The infected cell monolayers were fixed with 3.8% formalin and treated with 3% H_2O_2 in methanol for 5 min. Immunohistochemical staining was performed by using the Vectastain Elite kit (Vector, Burlingame, CA). The primary antibody used was the anti-p53 antibody PAb1801, and the secondary antibody was an avidin-labeled anti-mouse IgG (Vector). The biotinylated horseradish peroxidase avidin-biotin complex reagent was used to detect the antigen-antibody complex. Preadsorption controls were used in each immunostaining experiment. The cells were then counterstained with Harris hematoxylin (Sigma Chemical Co., St. Louis, MO).

Cell Growth Assay. Cells were plated at a density of 2 × 10^4 cells/ml in 6-well plates in triplicate. Cells were infected with either wild-type (Ad5CMV-p53) or replication-deficient adenovirus as a control. Cells were harvested every 2 days and counted; their viability was determined by trypan blue exclusion.

Inhibition of Tumor Growth *in Vivo*. The effect of Ad5CMV-p53 on established s.c. tumor nodules was determined in nude mice in a defined pathogen-free environment. Experiments were reviewed and approved by institutional committees for both animal care and use and for recombinant DNA research. Briefly, following induction of acepromazine/ketamine anesthesia, three separate s.c. flaps were elevated on each animal, and 5 × 10^6 cells in 150 μl of complete media were injected s.c. into each flap using a blunt needle; the cells were kept in the pocket with a horizontal mattress suture. Four animals were used for each cell line. After 4 days, the animals were reanesthetized, and the flaps were reelevated for the delivery of 100 μl of: (*a*) Ad5CMV-p53 (10^8 PFU) in the right anterior flap; (*b*) replication-defective virus (10^8 PFU) in the right posterior flap; and (*c*) transport medium alone, in the left posterior flank. All injection sites had developed s.c. visual and palpable nodules before treatment was administered. Animals were observed daily and sacrificed on day 20. *In vivo* tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of cross-sectional diameters. Following sacrifice, excised tumors were measured three dimensionally by microcalipers to determine tumor volume. A nonparametric Friedman's two-way analysis of variance test was used to test the significance of the difference between means of samples; the SPSS/PC+ software package (SPSS, Inc., Chicago, IL) was used.

Results

Adenoviral Infection of SCCHN Cells. The conditions for optimal adenoviral transduction of Tu-138 and Tu-177 cells were determined by infecting these cells with adenovirus expressing the *Escherichia coli* β-gal gene. The transduction efficiency was assessed by counting the number of blue cells after X-gal staining. There appeared to be a linear relationship between the number of infected cells and the number of adenovirus particles used. Cells inoculated with a single dose of 100 MOI β-gal adenovirus exhibited 60% blue cells (Fig. 1*A*), and this was improved to 100% by multiple infections (data not shown). The transduction efficiency of this vector in SCCHN cells is quite different from that of other cell lines examined previously; HeLa, HepG2, LM2, and human non-small cell lung cancer cell lines

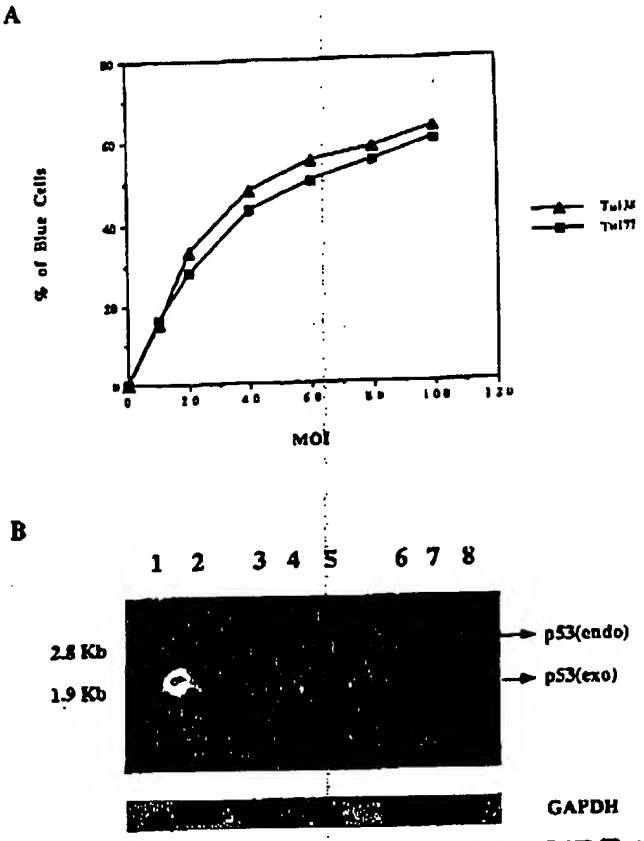

Fig. 1. *A*, transduction efficiency of SCCHN cell lines Tu-138 (▲) and Tu177 (■). A recombinant β-gal adenovirus was used to infect the cells at different MOIs ranging from 10 to 100. The percentages of β-gal-positive cells were obtained from scoring 500 cells each on replicate dishes. *B*, expression of exogenous *p53* mRNA 24 h after Ad5CMV-p53 infection. *Lanes 1* and *2*, 293 and K562 cells, respectively. *Lanes 3* and *6*, mock-infected Tu-138 and Tu-177 cells. *Lanes 4* and *7*, Tu-138 and Tu-177 cells infected with dl312. *Lanes 5* and *8*, Tu-138 and Tu-177 cells infected with Ad5CMV-p53.

showed 97 to 100% infection efficiencies after incubation with 30 to 50 MOI β-gal adenovirus (22).

Expression of Exogenous *p53* mRNA in Adenovirus-infected SCCHN Cells. Two human SCCHN cell lines were chosen for this study; both cell lines Tu-138 and Tu-177 possess a mutated *p53* gene (unpublished data). The recently created recombinant wild-type p53 adenovirus, Ad5CMV-p53, was used to infect Tu-138 and Tu-177 cells. Twenty-four h after infection, total RNA was isolated, and Northern blot analysis was performed. The transformed primary human embryonal kidney cell line 293 was used as a positive control because of its high level of expression of the *p53* gene product, whereas K562, a lymphoblastoma cell line with a homozygous deletion of the *p53* gene, was the negative control (Fig. 1*B*, *Lanes 1* and *2*, respectively). Due to unequal loading, only a fraction of the endogenous *p53* mRNA was detected in the 293 cells (Fig. 1*B*, *bottom panel*). The levels of the 2.8-kilobase endogenous *p53* mRNA detected in the samples isolated from mock-infected cells (Fig. 1*B*, *Lanes 3* and *6*) and from the cells infected with a replication-defective adenovirus, dl312 (Fig. 1*B*, *Lanes 4* and *7*), were similar. Up to 10-fold higher levels of exogenous 1.9-kilobase *p53* mRNA were present in the cells infected with Ad5CMV-p53 (Fig. 1*B*, *Lanes 5* and *8*), indicating that the exogenous *p53* cDNA was successfully transduced into these cells and efficiently transcribed. Interestingly, the level of endogenous *p53* mRNA in these cells was 5-fold higher than in the experimental controls. Northern blots exhibited no evidence of Ad5CMV-p53 (DNA) contamination of RNA.

Expression of p53 Protein in Adenovirus-infected SCCHN Cells. Western blot analysis was performed to compare the levels of *p53* mRNA to the amount of p53 protein produced. A p53 band, recognized by monospecific anti-p53 antibody, PAb1801, was observed in cellular extracts isolated from all samples except K562 cells (Fig. 2*A*, *Lane 8*). Cell line 293 showed high levels of p53 protein (Fig. 2*A*, *Lane 1*). Samples isolated from mock-infected Tu-138 and Tu-177 cells exhibited low levels of p53 protein (Fig. 2*A*, *Lanes 2* and *5*). The level of p53 expression remained similar in those cells infected with the dl312 adenovirus (Fig. 2*A*, *Lanes 3* and *6*). The levels of p53 antigen detected in Ad5CMV-p53-infected cells were significantly higher than the levels of the endogenous mutated proteins in both cell lines (Fig. 2*A*, *Lanes 5* and *7*). This result indicates that the exogenous *p53* mRNA produced from cells infected with Ad5CMV-p53 is efficiently translated into immunoreactive p53 protein. Furthermore, immunohistochemical analysis of cells infected with Ad5CMV-p53 revealed the characteristic nuclear staining of p53 protein (Fig. 2*B*, *right panel*), whereas mock-infected cells failed to show similar staining despite the presence of the p53 protein in these cells (Fig. 2*B*, *left panel*). This inability to detect the protein may be attributable to the insensitivity of the assay.

Effect of Exogenous p53 on SCCHN Cell Growth *in Vitro*. Cells infected with control virus dl312 had growth rates similar to those of the mock-infected cells (Fig. 3), whereas growth of the Ad5CMV-

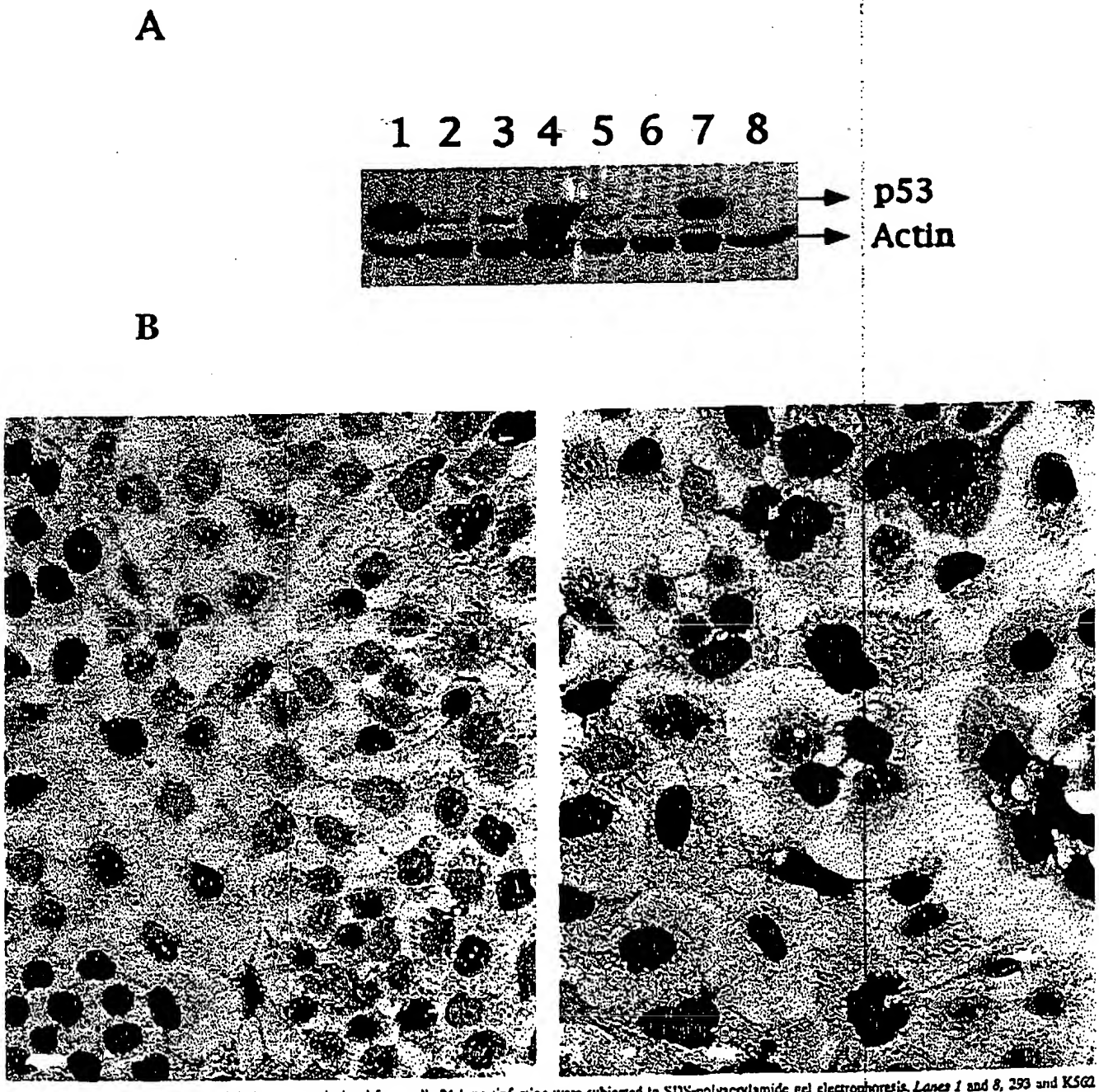

Fig. 2. *A*, Western blot analysis. Cellular extracts isolated from cells 24 h postinfection were subjected to SDS-polyacrylamide gel electrophoresis. *Lanes 1* and *8*, 293 and K562 cells, respectively. *Lanes 2* and *5*, mock-infected Tu-138 and Tu-177 cells. *Lanes 3* and *6*, Tu-138 and Tu-177 cells infected with dl312. *Lanes 4* and *7*, Tu-138 and Tu-177 cells infected with the Ad5CMV-p53. *B*, representative immunohistochemical staining of mock-infected Tu-138 cells (*left*) and Ad5CMV-p53-infected Tu-138 cells (*right*) 24-h postinfection. × 250.

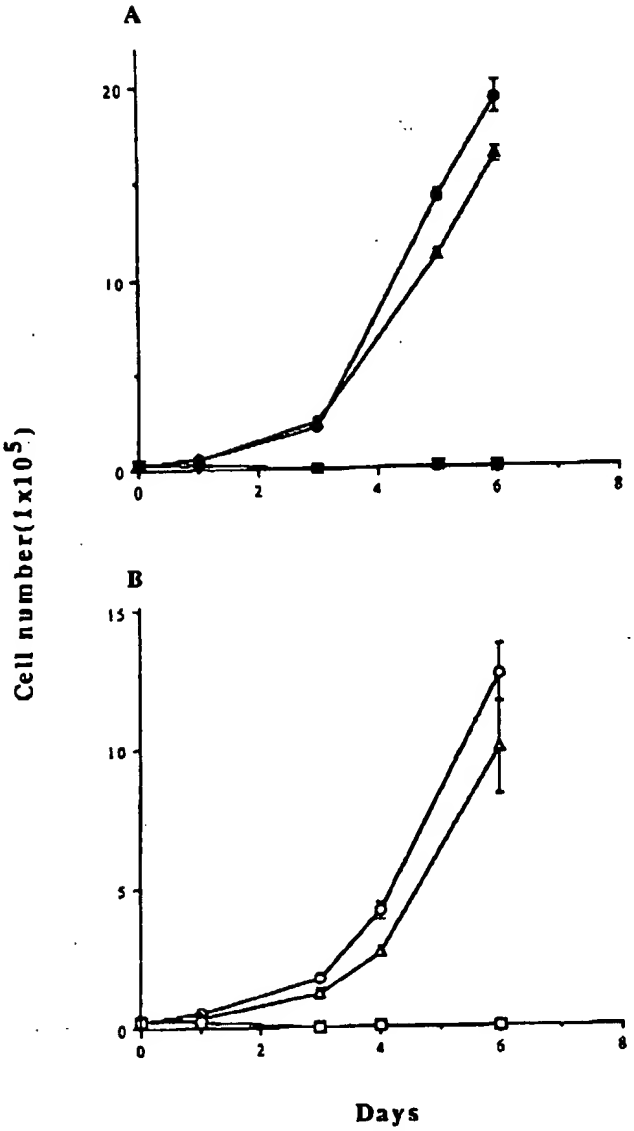

Fig. 3. Inhibition of SCCHN cell growth *in vitro*. *A*, growth curve of mock-infected Tu-138 cells (●), dl312-infected cells (▲), and Ad5CMV-p53-infected cells (■). *B*, growth curve of mock-infected Tu-177 cells (○), dl312-infected cells (△), and Ad5CMV-p53-infected cells (□). At each indicated time point, three dishes of cells were trypsinized and counted. The mean of cell counts per triplicate wells following infection were plotted against the number of days since infection; *bars*, SEM.

p53-infected Tu-138 (Fig. 3*A*) and Tu-177 (Fig. 3*B*) cells was greatly suppressed. Twenty-four h after infection, an apparent morphological change occurred with portions of the cell population rounding up and their outer membranes forming blebs. These are part of a series of histologically predictable events that constitute programmed cell death. The effect was more prominent for Tu-138 than for Tu-177 cells. Cells infected with the replication-defective adenovirus, dl312, demonstrated normal growth characteristics with no histomorphological abnormalities. Growth assays were reproducible in four repeated experiments.

Inhibition of Tumor Growth *in Vivo*. Seven animals were tested for each cell line. One animal in the Tu-177 group died following the second flap surgery and delivery of the therapeutic interventions, presumably due to profound anesthesia and subsequent mutilation by cage mates. Necropsy revealed no evidence of metastasis or systemic effects. Fig. 4 shows representative Tu-138 (*left*) and Tu-177 recipients (*right*). Sizable tumors are apparent on both posterior flaps of the animals (*i.e.*, the sites that did not receive Ad5CMV-p53). The lack of tumor progression is significant in the right anterior flaps of the animals which received Ad5CMV-p53 ($P < .04$). That Tu-177 cells have a slower growth rate has been established previously in these animals.[4] Two animals in the Tu-177 group had complete clinical and pathological regression of their established s.c. tumor nodule. Two animals in the Tu-138 group were killed early because they were experiencing rapid growth and ulceration of the control tumor sites. All surgical sites had developed lesions of at least 6 mm^3 before intervention. The tumor volumes on necropsy are shown in Table 1.

Discussion

Mutations or deletions of the *p53* tumor suppressor gene are the most frequent genetic alterations reported in SCCHN. Since the wild-type *p53* gene is believed to be involved primarily in delivering antiproliferative signals that may be capable of antagonizing the growth-stimulatory signals propagated by oncogene products, the potential molecular therapeutic effect of this gene in SCCHN deserves attention.

The rapid development in the field of gene therapy, including the creation of adenoviral vectors, has created an environment that is well suited for progress toward novel gene therapy of SCCHN. Because of their natural tropism for aerodigestive tract epithelium, adenoviruses may be uniquely suitable for the transient delivery of genes to cancers in these epithelial tissues. The recombinant, replication-defective adenoviruses that have been developed for gene therapy are missing the entire E1a and part of the E1b regions and are, therefore, capable of propagating only in cells that can provide the E1 proteins in trans, such as the 293 cell line. In the past few years, recombinant adenoviruses have been extensively developed and used for *in vivo* gene therapy. The high transfer efficiency of adenoviral vectors over a broad range of hosts both *in vitro* and *in vivo* make them attractive vehicles for molecular therapy. Recently, a recombinant wild-type p53 adenoviral vector (Ad5CMV-p53) was generated. This provided us with an excellent candidate for investigation of the biological effects of the wild-type *p53* gene product on SCCHN cells bearing the mutated *p53* gene. Using a β-gal recombinant adenovirus, the gene transfer efficiency of SCCHN cells was established. Approximately 60% of SCCHN cells were positive after X-gal staining. There appeared to be a linear correlation between the number of cells expressing the gene and the amount of viral particles used in the experiment. This result coincided with the efficiency obtained in cells infected with Ad5CMV-p53 after immunostaining by using a monoclonal anti-p53 antibody. Our observed transduction efficiency was lower than that achieved in other cell lines tested, including HeLa, HepG2, LM2, and the human non-small cell lung cancer cell lines. This discrepancy could be due to a host of factors, including receptor variations and differences in membrane characteristics among the cell lines. Additionally, the transduction efficiency of SCCHN cells may have been underestimated by limitations of light microscopic analyses.

Ad5CMV-p53 mediated a high level of expression of the *p53* gene in SCCHN cells. Two *p53* mRNA species were detected in the Ad5CMV-p53-infected cells. The high level of 1.9-kilobase mRNA was derived from the transduced *p53* cDNA following infection with Ad5CMV-p53, indicating that the adenoviral vector is an efficient vehicle for gene delivery into human SCCHN cells. Moreover, the levels of endogenous 2.8-kilobase mRNA were higher in the transduced cells than in the controls, presumably due to the effect of wild-type *p53* gene product. This phenomenon of transcriptional

[4] Unpublished data.

Fig. 4. Inhibition of SCCHN cell growth *in vivo*. Pictures of the representative nude mice studies for both Tu-138 (*left*) and Tu-177 (*right*) cell lines 20 days following therapeutic interventions. The right posterior flank received dl312, the left flank received transport medium alone, and the right anterior flap received Ad5CMV-p53, all 4 days following the establishment of a s.c. tumor.

Table 1 *Effect of Ad5CMV-p53 on tumor growth in nude mice*[a]

Treatment	Mean volume (mm^3 ± SEM) Tu-138 (7)	Tu-177 (6)
Ad5CMV-p53	22.3 ± 14	13 ± 18
Ad5(dl312)	803 ± 300	533 ± 148
Medium	1297 ± 511	421 ± 143
Significance	P	P
p53[b]:dl312	0.03	0.02
p53:medium	0.04	0.03

[a] The cells were injected s.c. at 5×10^6 cells/flap. Tumor sizes were determined at day 20 after treatment. Numbers in parentheses, the number of animals evaluated.
[b] Ad5CMV-p53 is abbreviated as p53; dl312 is an abbreviation for Ad5(dl312).

autoregulation of the *p53* gene has been well documented in murine cell lines in which the wild-type *p53* can transactivate its own promoter and the mutant *p53* fails to regulate the *p53* promoter (24).

Due to the episomal property of adenoviral vectors, all the input DNA following infection with Ad5CMV-p53 is presumably degraded slowly throughout incubation. By using polymerase chain reaction-based detection techniques, DNA can be detected as late as 14 days postinfection (data not shown).

Western blot analysis demonstrated that there were few or no changes of p53 protein levels between mock- and replication-defective adenovirus-infected cells, whereas production of p53 protein was significant in Ad5CMV-p53-infected cells, suggesting that the exogenous *p53* mRNA was efficiently translated. Time course protein expression studies have shown protein expression to peak 3 days postinfection and progressively decline to still detectable Western blotting levels on day 15 (22). Functionally, these SCCHN cells transduced with wild-type p53 gene exhibited significant inhibition of growth *in vitro* as compared to the mock-infected and replication-defective cells, thus clearly illustrating that these results were not mediated by the virus itself. The mechanism by which wild-type p53 protein inhibits growth *in vitro* may be related to arrest of the G_1 cell cycle (18), apoptosis (19, 20), or induction of another tumor suppressor gene such as *WAF1/CIP1* (25). The induction of apoptosis is one of the several documented functions of wild-type *p53*. When Tu-138 and Tu-177 cells were infected with Ad5CMV-p53 at 100 plaque-forming units/cell, the characteristic apoptotic histomorphology, such as rounded-up cells and the formation of blebs, was apparent as early as 4 h after infection and was followed rapidly by cell death (data not shown). However, the mechanism of growth suppression and cell death induced by Ad5CMV-p53 requires further investigation.

Encouraging results were also obtained in the nude mice studies. Tumor growth in the Ad5CMV-p53-infected cells was suppressed by at least 60 times more than in the experimental controls. These *in vivo* results confirmed the *in vitro* effects of Ad5CMV-p53 on human SCCHN cells, suggesting that the wild-type p53 protein mediates a potentially therapeutic effect. Although the *in vivo* studies are in their infancy, they nevertheless portend the development of a model for gene therapy in SCCHN that uses p53 adenovirus as a therapeutic intervention. Information derived from such studies could be expanded in the development of other novel molecular therapies that use adenoviral vectors, not only in SCCHN but in other human cancers. Several critical questions remain unanswered. How should the insult from antibodies that may arise in animals or patients following viral treatment be alleviated? How safe is this virus in humans? The results of the preliminary studies justify further investigation of *in vivo* animal models as well as mechanisms through which wild-type *p53* regulates these *in vitro* and *in vivo* effects.

References

1. Cancer Facts and Figures. Publication No. 90–425. M. No. 5008-LE. Washington, DC: American Cancer Society, 1990.
2. Thawley, S. E., and Panje, W. R. (eds.). Comprehensive Management of Head and Neck Tumors, Vol. 2, pp. 1158–1172. Philadelphia: W. B. Saunders, 1987.
3. van der Riet, P., Nawroz, H., Hruban, R. H., Corio, R., Tokino, K., Koch, W., and Sidransky, D. Frequent loss of chromosome 9p21–22 early in head and neck cancer progression. Cancer Res., *54:* 1156–1158, 1994.
4. Yamamoto, T., Kamata, N., Kawano, H., Shimizu, S., Kuroki, T., Toyoshima, K., Rikimaru, K., Nomura, N., Ishizaki, R., Pastan, I., Gamou, S., and Shimizu, N. High incidence of amplification of the epidermal growth factor receptor gene in human squamous carcinoma cell lines. Cancer Res., *46:* 414–416, 1986.
5. Somers, K. D., Cartwright, S. L., and Schechter, G. L. Amplification of the *int-2* gene in human head and neck squamous cell carcinomas. Oncogene, *5:* 915–920, 1990.
6. Berenson, R., Yang, J., and Mickel, R. A. Frequent amplification of the *bcl-1* locus in head and neck squamous cell carcinomas. Oncogene, *4:* 1111–1116, 1989.
7. Field, J. K., Spandidos, D. A., Stell, P. M., Vaughan, E. D., Evan, G. I., and Monro, J. P. Elevated expression of the c-myc oncoprotein correlates with poor prognosis in head and neck squamous cell carcinoma. Oncogene, *4:* 1463–1468, 1989.
8. Rodrigues, N. R., Rowan, A., Smith, M. E. F., Kerr, I. B., Bodmer, W. F., Gannon, J. V., and Lane, D. P. p53 mutations in colorectal cancer. Proc. Natl. Acad. Sci. USA, *87:* 7555–7559, 1990.
9. Bartek, J., Iggo, R., Gannon, J., and Lane, D. P. Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. Oncogene, *5:* 893–899, 1990.
10. Takahashi, T., Takahashi, T., Suzuki, H., Hida, T., Sekido, Y., Ariyoshi, Y., and Ueda, R. The *p53* gene is very frequently mutated in small-cell lung cancer with a distinct nucleotide substitution pattern. Cancer Res., *52:* 734–736, 1992.
11. Mashal, R., Shtalrid, M., Talpaz, M., Kantarjian, H., Smith, L., Beran, M., Cork, A., Trujillo, J., Gutterman, J., and Deisseroth, A. Rearrangement and expression of p53

in the chronic phase and blast crisis of chronic myelogenous leukemia. Blood, *75:* 180–189, 1990.

12. Maestro, R., Dolcetti, R., Gasparotto, D., Doglioni, C., Pelucchi, S., Barzan, L., Grandi, E., and Boiocchi, M. High frequency of *p53* gene alterations associated with protein overexpression in human squamous cell carcinoma of the larynx. Oncogene, *7:* 1159–1166, 1992.
13. Chung, K. Y., Mukhopadhyay, T., Kim, J., Casson, A., Ro, J. Y., Goepfert, H., Hong, W. K., and Roth, J. A. Discordant *p53* gene mutations in primary head and neck cancers and corresponding second primary cancers of the upper aerodigestive tract. *53:* 1676–1683, 1993.
14. Hoollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. p53 mutations in human cancers. Science (Washington DC), *253:* 49–53, 1991.
15. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K., and Vogelstein, B. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science (Washington DC), *249:* 912–915, 1990.
16. Mercer, W. E., Shields, M. T., Amin, M., Sauve, G. J., Appella, E., Romano, J. W., and Ullrich, S. J. Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. Proc. Natl. Acad. Sci. USA, *87:* 6166–6170, 1990.
17. Cai, D. W., Mukhopadhyay, T., Liu, Y., Fujiwara, T., and Roth, J. A. Stable expression of the wild-type *p53* gene in human lung cancer cells after retrovirus-mediated gene transfer. Hum. Gen. Ther., *4:* 617–624, 1993.
18. Diller, L., Kassel, J., Nelson, C. E., Gryka, M. A., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S. J., and Vogelstein, B. p53 functions as a cell cycle control protein in osteosarcomas. Mol. Cell. Biol., *10:* 5772–5781, 1990.
19. Shaw, P., Bovey, R., Tardy, S., Sahli, R., Sordat, B., and Costa, J. Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc. Natl. Acad. Sci. USA, *89:* 4495–4499, 1992.
20. Fujiwara, T., Grimm, E. A., Mukhopadhyay, T., Cai, D. W., Owen-Schaub, L. B., and Roth, J. A. A retroviral wild-type p53 expression vector penetrates human lung cancer spheroids and inhibits growth by inducing apoptosis. Cancer Res., *53:* 4129–4133, 1993.
21. Kozarsky, K. F., and Wilson, J. M. Gene therapy: adenovirus vectors. Curr. Opin. Gen. Dev., *3:* 499–503, 1993.
22. Zhang, W-W., Fang, X., Mazur, W., French, B. A., Georges, R. N., and Roth, J. A. High-efficiency gene transfer and high-level expression of wild-type p53 in human lung cancer cells mediated by recombinant adenovirus. Cancer Gen. Ther., *1:* 1–10, 1994.
23. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162:* 156–159, 1987.
24. Deffie, A., Wu, H., Reinke, V., and Lozano, G. The tumor suppressor p53 regulates its own transcription. Mol. Cell. Biol., *13:* 3415–3423, 1993.
25. El-Deiry, W. S., Harper, J. W., O'Connor, P. M., *et al.* WAF1/CIP1 is induced in p53-mediated G_1 arrest and apoptosis. Cancer Res., *54:* 1169–1174, 1994.

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Advances in Brief

Growth Suppression of Human Head and Neck Cancer Cells by the Introduction of a Wild-Type *p53* Gene via a Recombinant Adenovirus[1]

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Abstract

Mutations of the *p53* gene constitute one of the most frequent genetic alterations in squamous cell carcinoma of the head and neck (SCCHN). In this study, we introduced wild-type *p53* into two separate SCCHN cell lines via a recombinant adenoviral vector, Ad5CMV-p53. Northern blotting showed that following infection by the wild-type *p53* adenovirus (Ad5CMV-p53), cells produced up to 10-fold higher levels of exogenous *p53* mRNA than cells treated with vector only (without *p53*). Western blotting showed that the increased levels of p53 protein produced in the Ad5CMV-p53-infected cells were a reflection of *p53* mRNA expression. *In vitro* growth assays revealed growth arrest following Ad5CMV-p53 infection as well as cell morphological changes consistent with apoptosis. *In vivo* studies in nude mice with established s.c. squamous carcinoma nodules showed that tumor volumes were significantly reduced in mice that received peritumoral infiltration of Ad5CMV-p53. These data suggest that Ad5CMV-p53 may be further developed as a potential novel therapeutic agent for SCCHN since introduction of wild-type p53 into SCCHN cell lines attenuates their replication and tumor growth.

Introduction

Patients with SCCHN[3] are afflicted with a disease process that often has profound effects on speech, swallowing, and cosmesis. Furthermore, the overall rate of survival among these patients has remained unchanged at approximately 45% for nearly 30 years since contemporary surgery and radiation therapy were instituted (1). Treatment failures among these patients remain local and regional; only 10–15% of patients with the disease die of distant metastasis alone (2).

Although we have gained in understanding of the molecular events in the initiation and progression of SCCHN, they continue to require intensive investigation. A recent study identifying loss of heterozygosity of chromosome 9p21–22 as the most frequent genetic alteration in SCCHN suggested that this may be an early event in progression toward this neoplasm (3). Additionally, amplification and/or overexpression of cellular and nuclear oncogenes, such as c-*erB*-1 (4), *int*-2 (5), *bcl*-1 (6) and c-*myc* (7), have been documented in these cancers. The tumor suppressor gene *p53* has been the subject of immense interest and investigation in recent years. Alterations in the *p53* gene, including deletion, insertion, and point mutation, are the most frequent genetic events in many different carcinomas, such as those of the colon (8), breast (9), and lung (10), as well as soft-tissue sarcomas and leukemias (11). Several investigators have demonstrated the high frequency of *p53* gene alterations in SCCHN (12, 13).

There is considerable evidence implicating mutations of the *p53* gene in the etiology of many human cancers (14). Reports have demonstrated that growth of several different human cancer cell lines, including representatives of colon cancer (15), glioblastoma (16), breast cancer (17), and osteosarcoma (18), can be functionally suppressed by DNA transfection or retrovirus-mediated transfer of the wild-type *p53* gene. This gene may have an important role not only in cell growth but in apoptosis (programmed cell death). Induction of exogenous expression of wild-type *p53* has been shown to induce apoptosis in colon cancer cell lines (19) and in human lung cancer spheroids (20).

The adenoviral vector has emerged as a leading candidate for *in vivo* gene therapy in the past few years. It enjoys an advantage over traditional DNA transfection and retroviral transfer in its high efficiency of transferring potentially therapeutic genes into a wide range of host cells (21). The recently created adenoviral vector containing wild-type *p53* (Ad5CMV-p53; Ref. 22) provides us with an attractive delivery system to investigate the effect of exogenous wild-type *p53* on SCCHN cell lines both *in vitro* and *in vivo*. The outcome of this study indicates that further development of the p53 adenovirus or other novel molecular therapies for SCCHN is warranted.

Materials and Methods

Cell Lines and Culture Conditions. Human SCCHN cell lines Tu-138 and Tu-177 were both established at the Department of Head and Neck Surgery, M. D. Anderson Cancer Center. Tu-138 and Tu-177 were established from a gingivo-labial moderately differentiated squamous carcinoma and a poorly differentiated squamous carcinoma of the larynx, respectively. Both cell lines were developed via primary explant technique and are cytokeratin positive and tumorigenic in athymic nude and SCID mice. These cells were grown in DMEM/F12 medium supplemented with 10% heat-inactivated FBS with penicillin/streptomycin.

Recombinant Adenovirus Preparation and Infection. The recombinant p53 adenovirus (Ad5CMV-p53; Ref. 22) contains the CMV promoter, wild-type p53 cDNA, and SV40 polyadenylation signal in a minigene cassette inserted into the E1-deleted region of modified Ad5. Viral stocks were propagated in 293 cells. Cells were harvested 36–40 h after infection, pelleted, resuspended in phosphate-buffered saline, and lysed; cell debris was removed by subjecting the cells to CsCl gradient purification. Concentrated virus was dialyzed, aliquoted, and stored at −80°C. Infection was carried out by the addition of the virus to the DMEM/F12 medium and 2% FBS to the cell monolayers. The cells were incubated at 37°C for 60 min with constant agitation. Then complete medium (DMEM/F12–10% FBS) was added, and the cells were incubated at 37°C for the desired length of time.

Northern Blot Analysis. Total RNA was isolated by the acid-guanidinium thiocyanate method of Chomczynski and Sacchi (23). Northern analyses were performed on 20 μg of total RNA. The membrane was hybridized with a *p53*

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[3] The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; DMEM/F12, Dulbecco's modified Eagle's medium/Ham's F-12 medium; FBS, fetal bovine serum; Ad5, adenovirus 5; CMV, cytomegalovirus; Ad5CMV-p53, wild-type p53 adenovirus; cDNA, complementary DNA; MOI, multiplicity of infection; SDS, sodium dodecyl sulfate; β-gal, β-galactosidase; dl312, replication-defective adenovirus; PFU, plaque forming units.

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Development and Characterization of Recombinant Adenoviruses Encoding Human p53 for Gene Therapy of Cancer

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ABSTRACT

We have constructed recombinant human adenoviruses that express wild-type human p53 under the control of either the Ad 2 major late promoter (MLP) or the human cytomegalovirus (CMV) immediate early gene promoter. Each construct replaces the Ad 5 E1a and E1b coding sequences necessary for viral replication with the p53 cDNA and MLP or CMV promoter. These p53/Ad recombinants are able to express p53 protein in a dose-dependent manner in infected human cancer cells. Tumor suppressor activity of the expressed p53 protein was assayed by several methods. [^{3}H]Thymidine incorporation assays showed that the recombinant adenoviruses were capable of inhibiting DNA synthesis in a p53-specific, dose-dependent fashion. *Ex vivo* treatment of Saos-2 tumor cells, followed by injection of the treated cells into nude mice, led to complete tumor suppression using the MLP/p53 recombinant. Following a single injection of CMV/p53 recombinant adenovirus into the peritumoral space surrounding an *in vivo* established tumor derived from a human small cell lung carcinoma cell line (NIH-H69), we were able to detect p53 mRNA in the tumors at 2 and 7 days post-injection. Continued treatment of established H69 tumors with MLP/p53 recombinant led to reduced tumor growth and increased survival time compared to control treated animals. These results indicate that recombinant adenoviruses expressing wild-type p53 may be useful vectors for gene therapy of human cancer.

OVERVIEW SUMMARY

Introduction of the p53 tumor suppressor gene into tumors bearing p53 mutations can inhibit cellular proliferation and tumorigenicity. Wills *et al.* describe replication-deficient recombinant adenoviruses directing expression of human p53 both *in vitro* and *in vivo*. They show that adenovirus-mediated expression of wild-type p53 in p53 altered tumors can suppress proliferation and inhibit tumorigenicity in *ex vivo* and *in vivo* cancer models.

INTRODUCTION

MUTATION OF THE P53 GENE is the most common genetic alteration in human cancers (Bartek *et al.*, 1991; Hollstein *et al.*, 1991). In its proposed role as a "guardian of the genome" (Lane, 1992), the p53 gene product functions as a transcriptional activator of other genes which inhibit cell cycle progression from G_1 to S phase in normal cells. Its levels rise and accumulate in response to DNA damage, leading either to G_1 arrest and repair, terminal differentiation, or, if too much damage has occurred, apoptosis (Kuerbitz *et al.*, 1992; Lane, 1992). Loss of wild-type p53 function is associated with the uncontrolled growth of many types of human cancers. The reexpression of normal p53 in p53-altered tumor cells has been demonstrated to suppress tumor growth (Chen *et al.*, 1990; Cheng *et al.* 1992; Takahashi *et al.*, 1992) or induce apoptosis (Yonish-Rouach *et al.*, 1991, Shaw *et al.*, 1992). Therefore, p53 functions as a tumor suppressor, restoring a nontumorigenic phenotype to tumor cells in which the endogenous p53 gene has been deleted or mutated.

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Recent work has shown that human adenoviruses can be used to deliver genes successfully into a variety of cells and tissues (Lemarchand *et al.*, 1992; Rosenfeld *et al.*, 1992; Rich *et al.*, 1993). Recombinant adenoviruses have several advantages over alternative gene delivery systems such as retrovirus (RV) or adeno-associated virus (AAV)-based vectors for the treatment of cancer. These include the ability to produce stable, high-titer virus capable of efficient infection and subsequent gene expression in target cells (for review, see Siegfried, 1993). Because of the advantages of an adenovirus-based delivery system over other systems for the potential gene therapy of cancer, we constructed recombinant adenoviruses encoding wild-type p53 under the control of the Ad 2 major late promoter (MLP) or the human cytomegalovirus (CMV) promoter. We have tested the ability of these constructs to suppress tumor growth both *in vitro* and *in vivo*.

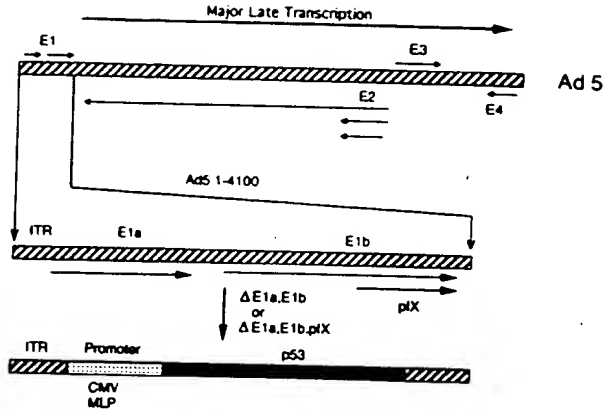

FIG. 1. Schematic of recombinant p53/adenovirus constructs. The p53 recombinants are based on Ad 5 and have had the E1 region of nucleotides 360–3,325 replaced with a 1.4-kb full-length p53 cDNA driven by the Ad 2 MLP (A/M/53) or human CMV (A/C/53) promoters followed by the Ad 2 tripartite leader cDNA. The control virus A/M has the same Ad 5 deletions as the A/M/53 virus, but lacks the 1.4-kb p53 cDNA insert. The remaining E1b sequence (705 nucleotides) have been deleted to create the protein IX-deleted constructs A/M/N/53 and A/C/N/53. These constructs also have a 1.9-kb *Xba* I deletion within adenovirus type 5 region E3.

MATERIALS AND METHODS

Cell lines

Recombinant adenoviruses were grown and propagated in the human embryonal kidney cell line 293 (ATCC CRL 1573) maintained in DME medium containing 10% defined, supplemented calf serum (Hyclone). Saos-2 cells were maintained in Kaighn's media supplemented with 15% fetal calf serum. HeLa and Hep 3B cells were maintained in DME medium supplemented with 10% fetal calf serum. All other cell lines were grown in Kaighn's media supplemented with 10% fetal calf serum. Saos-2 cells were kindly provided by Dr. Eric Stanbridge. All other cell lines were obtained from ATCC.

Construction of recombinant adenoviruses

To construct the Ad5/p53 viruses, a 1.4-kb *Hind* III–*Sma* I fragment containing the full-length cDNA for p53 was isolated from pGEM1-p53-B-T (kindly supplied by Dr. Wen-Hwa Lee) and inserted into the multiple cloning site of the expression vector pSP72 (Promega) using standard cloning procedures (Sambrook *et al.*, 1989). The p53 insert was recovered from this vector following digestion with *Xho* I–*Bgl* II and gel electrophoresis. The p53 coding sequence was then inserted into either pNL3C or pNL3CMV adenovirus gene transfer vectors (kindly provided by Dr. Robert Schneider), which contain the Ad5 5′ inverted terminal repeat and viral packaging signals and the E1a enhancer upstream of either the Ad2 major late promoter (MLP) or the human cytomegalovirus immediate early gene promoter (CMV), followed by the tripartite leader cDNA and Ad 5 sequence 3,325–5,525 bp in a pML2 background. These new constructs replace the E1 region (bp 360–3,325) of Ad5 with p53 driven by either the Ad2 MLP (A/M/53) or the human CMV promoter (A/C/53), both followed by the tripartite leader cDNA (see Fig. 1). The p53 inserts use the remaining downstream E1b polyadenylation site. Additional MLP- and CMV-driven p53 recombinants (A/M/N/53, A/C/N/53) were generated which had a further 705-nucleotide deletion of Ad 5 sequence to remove the protein IX (pIX) coding region. As a control, a recombinant adenovirus was generated from the parental pNL3C plasmid without a p53 insert (A/M). A second control (kindly provided by Dr. Robert Schneider) consisted of a recombinant adenovirus encoding the β-galactosidase (β-Gal) gene under the control of the CMV promoter (A/C/β-Gal). The plasmids were linearized with either *Nru* I or *Eco* RI and cotransfected with the large fragment of a *Cla* I-digested Ad 5 *dl*309 or *dl*327 mutants (Jones and Shenk, 1979; Thimmappaya *et al.*, 1982) using a Ca/PO_4 transfection kit (Strategene). Only the pIX-minus constructs used the *dl*327 background which contains a 1.9-kb *Xba* I deletion in the E3 region. Viral plaques were isolated and recombinants identified by both restriction digest analysis and the polymerase chain reaction (PCR) using recombinant-specific primers against the tripartite leader cDNA sequence with downstream p53 cDNA sequence. Recombinant virus was further purified by limiting dilution, and virus particles were purified and titered by standard methods (Graham and van der Erb, 1973; Graham and Prevec, 1991).

p53 protein detection

Saos-2 or Hep 3B cells (5×10^5) were infected with the indicated recombinant adenoviruses for a period of 24 hr at increasing multiplicities of infection (moi) of plaque-forming units of virus/cell. Purified adenovirus, stored in1% sucrose in phosphate-buffered saline (PBS), is diluted with media to obtain the desired moi and added to plates of cells containing fresh media. After 24 hr, the cells were washed once with PBS and harvested in lysis buffer [50 m*M* Tris-HCl pH 7.5, 250 m*M* NaCl, 0.1% NP-40, 50 m*M* NaF, 5 m*M* EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 m*M* phenylmethylsulfonyl fluoride (PMSF)]. A Bradford assay (Bio-Rad Protein Assay kit) was used to measure cellular protein concentration, and equal amounts of protein (approximately 30 μg) were separated

by 10% SDS-PAGE and transfered to nitrocellulose. Membranes were incubated with α-p53 antibody PAb 1801 (Novocastro) followed by sheep anti-mouse IgG conjugated with horseradish peroxidase. p53 protein was visualized by chemiluminescence (ECL kit, Amersham) on Kodak XAR-5 film.

Measurement of DNA synthesis rate

Cells (5×10^3/well) were plated in 96-well titer plates (Costar) and allowed to attach overnight (37°C, 7% CO_2). Cells were then infected for 24 hr with purified recombinant virus particles at moi values ranging from 0.3 to 100, as indicated. Media were changed 24 hr after infection, and incubation was continued for a total of 72 hr. [^{3}H]Thymidine (Amersham, 1 μCi/well) was added 18 hr prior to harvest. Cells were harvested on glass fiber filters and levels of incorporated radioactivity were measured in a beta scintillation counter. [^{3}H]Thymidine incorporation was expressed as the mean % (±SD) of media control and plotted versus the moi.

Tumorigenicity in nude mice

Approximately 2.4×10^8 Saos-2 cells, plated in T225 flasks, were treated with suspension buffer (1% sucrose in PBS) containing either A/M/N/53- or A/M-purified virus at an moi of 3 or 30. Following an overnight infection, cells were injected subcutaneously into the left and right flanks of BALB/c athymic nude mice (4 mice per group). One flank was injected with the A/M/N/53-treated cells, while the contralateral flank was injected with the control A/M-treated cells, each mouse serving as its own control. Animals receiving bilateral injection of buffer-treated cells served as additional controls. Tumor dimensions (length, width, and height) and body weights were then measured twice per week over an 8-week period. Tumor volumes were estimated for each animal, assuming a spherical geometry with radius equal to one-half the average of the measured tumor dimensions.

Intratumoral RNA analysis

Female BALB/c athymic nude mice (approximately 5 weeks of age) were injected subcutaneously with 1×10^7 H69 small cell lung carcinoma (SCLC) cells in a 200-μl volume in their right flanks. Tumors were then allowed to progress for 32 days. Mice then received peritumoral injections of either A/C/53 or A/C/β-Gal recombinant adenovirus [2×10^9 plaque-forming units (pfu)] into the subcutaneous space beneath the tumor mass. Tumors were excised from the animals 2 and 7 days post adenovirus treatment and rinsed with PBS. Tumor samples were homogenized, and total RNA was isolated using a TriReagent kit (Molecular Research Center, Inc.). Poly(A) RNA was isolated using the PolyATract mRNA Isolation System (Promega), and approximately 10 ng of sample was used for reverse transcriptase (RT)-PCR determination of recombinant p53 mRNA expression (Wang *et al.*, 1989). Primers were designed to amplify sequence between the adenovirus tripartite leader cDNA and the downstream p53 cDNA, ensuring that only recombinant, and not endogenous p53 would be amplified.

p53 gene therapy of established tumors in nude mice

Approximately 1×10^7 H69 (SCLC) tumor cells in 200-μl volumes were injected subcutaneously into female BALB/c athymic nude mice. Tumors were allowed to develop for 2 weeks, at which point animals were randomized by tumor size (n = 5/group). Peritumoral injections of either A/M/N/53 or the control A/M adenovirus (2×10^9 pfu/injection) or buffer alone (1% sucrose in PBS) were administered twice per week for a total of 8 doses/animal per group. Tumor dimensions and body weights were measured twice per week for 7 weeks, and tumor volume was estimated as described previously. Animals were then followed to observe the effect of treatment on mouse survival.

RESULTS

Construction of recombinant p53-adenovirus

p53 adenoviruses were constructed by replacing a portion of the E1a and E1b region of adenovirus type 5 with p53 cDNA under the control of either the Ad2 MLP (A/M/53) or CMV (A/C/53) promoter (schematized in Fig. 1). This E1 substitution severely impairs the ability of the recombinant adenoviruses to replicate, restricting their propagation to 293 cells that supply Ad 5 E1 gene products *in trans* (Graham *et al.*, 1977). After identification of p53 recombinant adenovirus by both restriction digest and PCR analysis, the entire p53 cDNA sequence from one of the recombinant adenoviruses (A/M/53) was sequenced to verify that it was free of mutations. Following this, purified preparations of the p53 recombinants were used to infect HeLa cells to assay for the presence of phenotypically wild-type adenovirus. HeLa cells, which are nonpermissive for replication of E1-deleted adenovirus, were infected with $1–4 \times 10^9$ infectious units of recombinant adenovirus at an moi = 50, cultured for 3 weeks, and observed for the appearance of cytopathic effect (CPE). Using this assay, we were not able to detect recombinant adenovirus replication or wild-type contamination, readily evident by the CPE observed in control cells infected with wild-type adenovirus at a level of sensitivity of approximately 1 in 10^9.

p53 protein expression from recombinant adenovirus

To determine if our p53 recombinant adenoviruses expressed p53 protein, we infected tumor cell lines that do not express endogenous p53 protein. The human tumor cell lines Saos-2 (osteosarcoma) and Hep 3B (hepatocellular carcinoma), which contain mutations that result in no expression of p53 protein (Chen *et al.*, 1990; Hsu *et al.*, 1993), were infected for 24 hr with the p53 recombinant adenoviruses A/M/53 or A/C/53 at moi values ranging from 0.1 to 200 pfu/cell. Western analysis of lysates prepared from infected cells demonstrated a dose-dependent p53 protein expression in both cell types (Fig. 2). Both cell lines expressed higher levels of p53 protein following infection with A/C/53 than with A/M/53 (Fig. 2). No p53 protein was detected in noninfected cells. Cells infected with moi values of up to 200 of the control virus A/M also did not show detectable p53 protein (unpublished observation). SW 480 cell

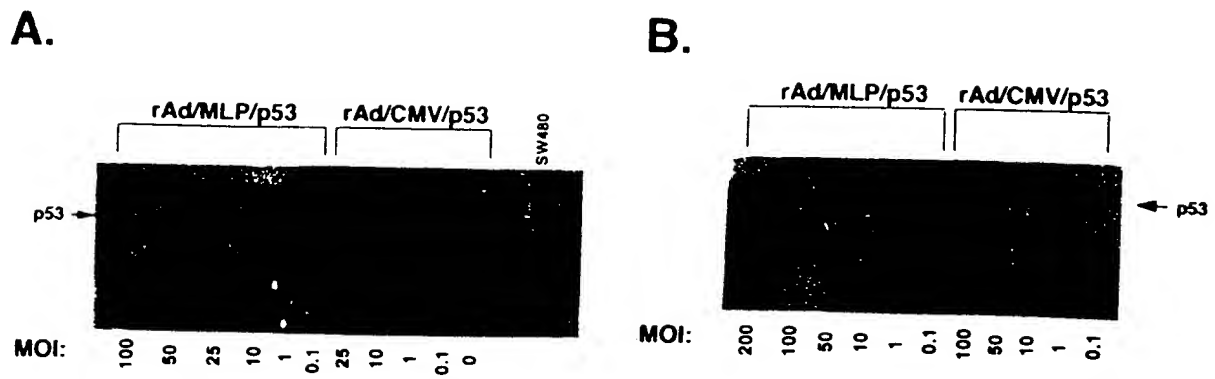

FIG. 2. p53 protein expression in tumor cells infected with A/M/53 and A/C/53. A. Saos-2 (osteosarcoma) cells were infected at the indicated moi with either the A/M/53- or A/C/53-purified virus and harvested 24 hr later. The p53 antibody pAb 1801 was used to stain immunoblots of samples loaded at equal total protein concentrations. Equal protein concentrations of SW480 cell extracts, which overexpress mutant p53 protein, were used as a marker for p53 size. The zero (0) under the A/C/53 heading indicates a mock infection containing untreated Saos-2 lysate. B. Hep 3B (hepatocellular carcinoma) cells were infected with the A/M/53 or A/C/53 virus at the indicated moi and analyzed as in A. The arrow indicates the position of the p53 protein.

lysate, which overexpresses mutant p53 protein (Baker *et al.*, 1990), was used as a size marker. Levels of endogenous wild-type p53 are normally quite low, and nearly undetectable by Western analysis of cell extracts (Bartek *et al.*, 1991). It is clear however that wild-type p53 protein levels are easily detectable after infection with either A/M/53 or A/C/53 at the lower moi values (Fig. 2), suggesting that even low doses of p53 recombinant adenoviruses can produce potentially efficacious levels of p53.

p53-dependent morphology changes

The reintroduction of wild-type p53 into the p53-negative osteosarcoma cell line, Saos-2, results in a characteristic enlargement and flattening of these normally spindle-shaped cells (Chen *et al.*, 1990). Subconfluent Saos-2 cells (1×10^5 cells/10-cm plate) were infected at an moi of 50 with either the A/C/53 or control A/M virus, and incubated at 37°C for 72 hr until uninfected control plates were confluent. At this point, the expected morphological change was evident in the A/C/53-treated plate (Fig. 3C), but not in uninfected (Fig. 3A) or control virus-infected plates (Fig. 3B). This effect was not a function of cell density because a control plate initially seeded at lower density retained normal morphology at 72 hr when its confluence approximated that of the A/C/53-treated plate (data not shown). Our previous results had demonstrated a high level of p53 protein expression at a moi of 50 in Saos-2 cells (Fig. 2A), and these results provided evidence that the p53 protein expressed by these recombinant adenoviruses was biologically active.

p53 inhibition of cellular DNA synthesis

To test further the activity of the p53 recombinant adenoviruses, we assayed their ability to inhibit proliferation of human tumor cells as measured by the uptake of [^{3}H]thymidine. It has previously been shown that introduction of wild-type p53 into cells that do not express endogenous wild-type p53 can arrest the cells at the G_1/S transition, leading to inhibition of uptake of labeled thymidine into newly synthesized DNA (Baker *et al.*, 1990; Diller *et al.*, 1990; Mercer *et al.*, 1990). We infected a variety of p53-deficient tumor cell lines with either A/M/N/53, A/C/N/53 or a non-p53-expressing control recombinant adenovirus (A/M). We observed a strong, dose-dependent inhibition of DNA synthesis by both the A/M/N/53 and A/C/N/53 recombinants in 7 out of the 9 different tumor cell lines tested (Fig. 4). Both constructs were able to inhibit DNA synthesis specifically in these human tumor cells, regardless of whether they ex-

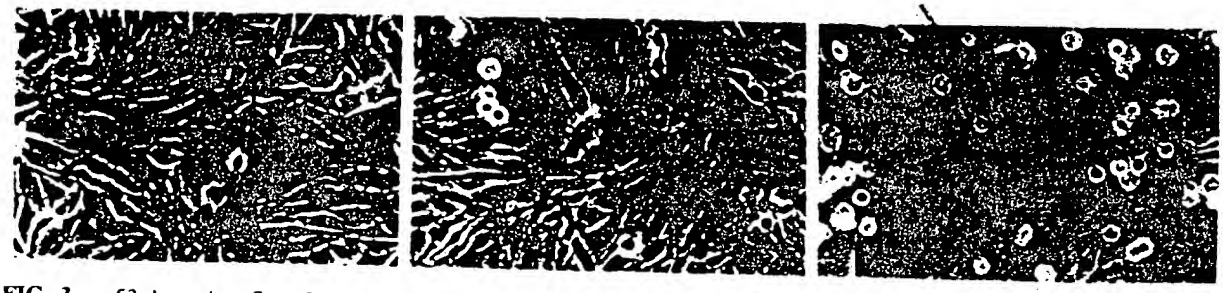

FIG. 3. p53-dependent Saos-2 morphology change. Subconfluent (1×10^5 cells/10-cm plate) Saos-2 cells were either uninfected (A), infected at a moi = 50 with the control A/M virus (B), or the A/C/53 virus (C). The cells were photographed 72 hr post-infection.

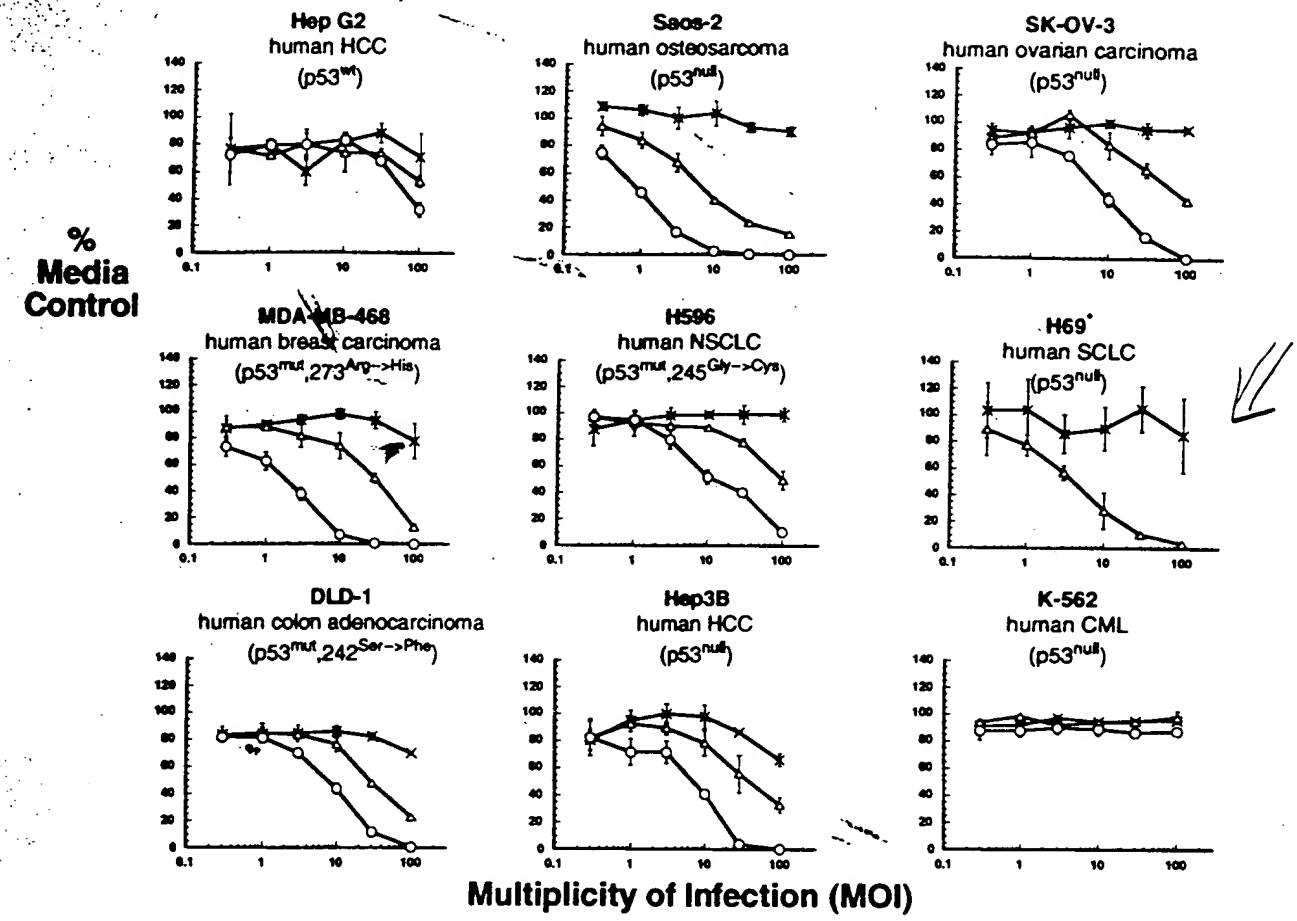

FIG. 4. p53-dependent inhibition of DNA synthesis in human tumor cell lines by A/M/N/53 and A/C/N/53. Nine different tumor cell lines were infected with either control adenovirus A/M/ (xx), or the p53-expressing A/M/N/53 (Δ) or A/C/N/53 (O) virus at increasing moi as indicated. Tumor type and p53 status are noted for each cell line (wt, wild type; null, no protein expressed; mut, mutant protein expressed). DNA synthesis was measured 72 hr post-infection as described in Materials and Methods. Results are from triplicate measurements at each dose (mean ± SD), and are plotted as % of media control *versus* moi. (*) H69 cells were only tested with A/M and A/M/N/53 virus.

pressed mutant p53 or failed to express p53 protein. We also found that in this assay, the A/C/N/53 construct was consistently more potent than the A/M/N/53. In Saos-2 (osteosarcoma) and MDA-MB468 (breast cancer) cells, nearly 100% inhibition of DNA synthesis was achieved with the A/C/N/53 construct at a moi as low as 10. At doses where inhibition by the control adenovirus is only 10–30%, we observed a 50–100% reduction in DNA synthesis using either p53 recombinant adenovirus. In contrast, we observed no significant p53-specific effect with either construct as compared to control virus in HEP G2 cells (hepatocarcinoma cell line expressing endogenous wild-type p53; Bressac *et al.*, 1990), nor in the K562 (p53 null; Feinstein *et al.*, 1992) leukemic cell line.

Tumorigenicity in nude mice

In a more stringent test of function for our p53 recombinant adenoviruses, we infected tumor cells *ex vivo* and then injected the cells into nude mice to assess the ability of the recombinants to suppress tumor growth *in vivo*. Saos-2 cells infected with A/M/N/53 or control A/M virus at a moi of 3 or 30 were injected into opposite flanks of nude mice. Tumor sizes were then measured twice a week over an 8-week period. At a moi of 30, we did not observe any tumor growth in the p53-treated flanks in any of the animals, while the control treated tumors continued to grow (Fig. 5). The progressive enlargement of the control virus-treated tumors was similar to that observed in the buffer-treated control animals. We also observed a clear difference in tumor growth between the control adenovirus and the p53 recombinant at a moi of 3, although tumors from 2 out of the 4 p53-treated mice did start to show some growth after approximately 6 weeks (data not shown). Thus, the A/M/N/53 recombinant adenovirus is able to mediate p53-specific tumor suppression in an *in vivo* environment. We have also observed very similar results when infecting and injecting the NSCLC cell line H596, which expresses mutant p53 protein with the same viruses (unpublished observations).

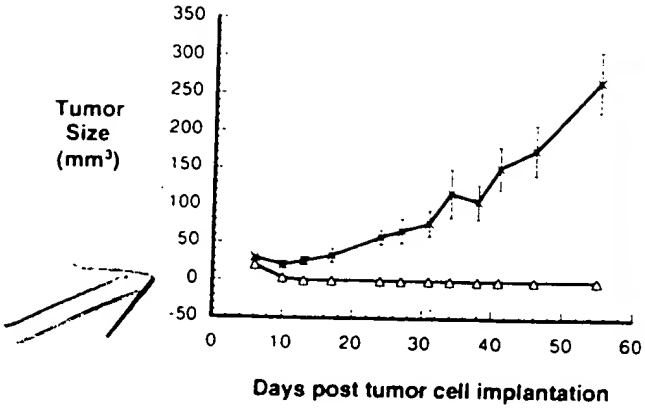

FIG. 5. Tumorigenicity of p53-infected Saos-2 cells in nude mice. Saos-2 cells were infected with either the control A/M virus or the p53 recombinant A/M/N/53 at moi = 30. Treated cells were injected subcutaneously into the flanks of nude mice, and tumor dimensions were measured (as described in Materials and Methods) twice per week for 8 weeks. Results are plotted as tumor size *versus* days post tumor cell implantation for both control A/M-(x) and A/M/N/53-(Δ) treated cells. Error bars represent the mean tumor size ± SEM for each group of 4 animals at each time point.

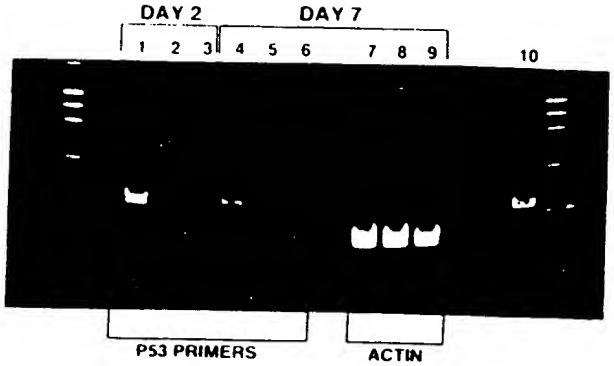

FIG. 6. Expression of rAd/p53 RNA in established tumors. H69 (SCLC) cells were injected subcutaneously into nude mice and allowed to develop tumors for 32 days until reaching a size of approximately 25–50 mm^3. Mice were randomized and injected peritumorally with 2×10^9 pfu of either control A/C/β-Gal or A/C/53 virus. Tumors were excised 2 and 7 days post injection, and poly(A) RNA was prepared from each tumor sample. RT-PCR was carried out using equal RNA concentrations and primers specific for recombinant p53 message. PCR amplification was for 30 cycles at 94°C 1 min, 55°C 1.5 min, 72°C 2 min, and a 10-min, 72°C final extension period in an Omnigen thermalcycler (Hybaid). The PCR primers used were a 5′ Tripartite Leader cDNA (5′-CGCCACCGAGGGACCTGAGCGAGTC-3′) and a 3′ p53 primer (5′-TTCTGGGAAGGGACAGAAGA-3′). Lanes 1, 2, 4, and 5, p53-treated samples excised at days 2 or 7 as indicated; lanes 3 and 6, from β-Gal-treated tumors; lanes 7, 8, and 9, replicates of lanes 4, 5, and 6, respectively, amplified with actin primers to verify equal loading; lane 10, a positive control using a tripartite/p53 containing plasmid.

In vivo *expression of rAd/p53*

Although *ex vivo* treatment of cancer cells and subsequent injection into animals provided a critical test of tumor suppression, a more clinically relevant experiment is to determine if injected p53 recombinant adenovirus could infect and express p53 in established tumors *in vivo*. To address this, H69 (SCLC, $p53^{null}$) cells were injected subcutaneously into nude mice, and tumors were allowed to develop for 32 days. At this time, a single injection of 2×10^9 pfu of either A/C/53 or A/C/β-Gal adenovirus was injected into the peritumoral space surrounding the tumor. Tumors were then excised at either day 2 or day 7 following the adenovirus injection, and poly(A) RNA was isolated from each tumor. RT-PCR, using recombinant-p53 specific primers, was then used to detect p53 mRNA in the p53-treated tumors (Fig. 6, lanes 1, 2, 4, 5). No p53 signal was evident from the tumors excised from the β-Gal-treated animals (Fig. 6, lanes 3 and 6). Amplification with actin primers served as a control for the RT-PCR reaction (Fig. 6, lanes 7–9), while a plasmid containing the recombinant-p53 sequence served as a positive control for the recombinant-p53-specific band (Fig. 6, lane 10). This experiment demonstrates that a p53 recombinant adenovirus can specifically direct expression of p53 mRNA within established tumors following a single injection into the peritumoral space. It also provides evidence for *in vivo* viral persistence for at least 1 week following infection with a p53 recombinant adenovirus.

In vivo efficacy

To address the feasibility of gene therapy of established tumors, a tumor-bearing nude mouse model was used. H69 cells were injected into the subcutaneous space on the right flank of mice, and tumors were allowed to grow for 2 weeks. Mice then received peritumoral injections of buffer or recombinant virus twice weekly for a total of 8 doses. In the mice treated with buffer or control A/M virus, tumors continued to grow rapidly throughout the treatment, whereas those treated with the A/M/N/53 virus grew at a greatly reduced rate (Fig. 7A). Although control animals treated with buffer alone had accelerated tumor growth as compared to either virus-treated group, we found no significant differences in body weight among the three groups during the treatment period (data not shown). Tumor ulceration in some animals limited the relevance of tumor size measurements after day 42. However, continued monitoring of the animals to determine survival time demonstrated a survival advantages for the p53-treated animals (Fig. 7B). The last of the control adenovirus-treated animals died on day 83, while buffer alone treated controls had all expired by day 56. In contrast, all 5 animals treated with the A/M/N/53 survived up to day 137 before the first animal in this group died (Fig. 7B). Two animals continue to survive at day 174. Together, our data indicate a p53-specific effect on both tumor growth and survival time in animals with established p53-deficient tumors.

DISCUSSION

Adenovirus vectors expressing p53

We have constructed recombinant human adenovirus vectors that are capable of expressing high levels of wild-type p53

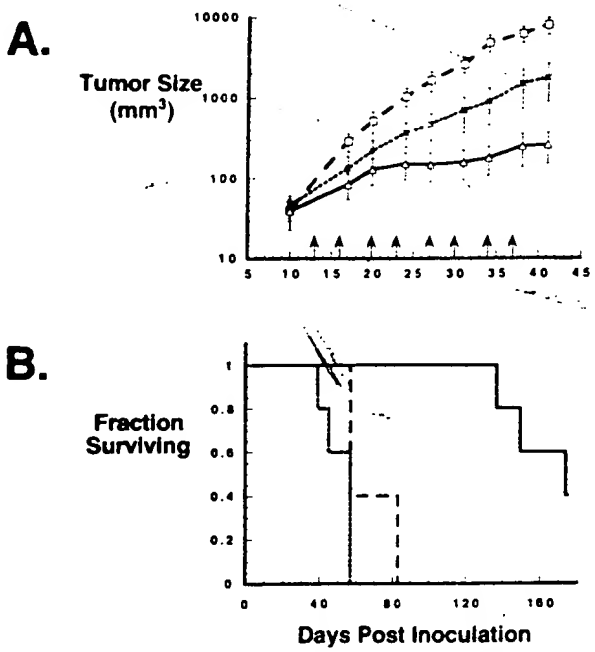

FIG. 7. *In vivo* tumor suppression and increased survival time with A/M/N/53. H69 (SCLC) tumor cells were injected subcutaneously into nude mice and allowed to develop for 2 weeks. Peritumoral injections of either buffer alone (□), control A/M adenovirus (x), or A/M/N/53 (Δ) (both virus 2×10^9 pfu/injection) were administered twice per week for a total of 8 doses. Tumor dimensions were measured twice per week and tumor volume was estimated as described in Materials and Methods. A. Tumor size is plotted for each virus *versus* time (days) post inoculation of H69 cells. Error bars indicate the mean tumor size ± SEM for each group of 5 animals. Arrows indicate days of virus injections. B. Mice were monitored for survival and the fraction of mice surviving per group *versus* time post inoculation of buffer alone (—), control A/M (— —), or A/M/N/53 (——) virus-treated H69 cells is plotted.

protein in a dose-dependent manner. Each vector contains deletions in the E1a and E1b regions that render the virus replication deficient (Challberg and Kelly, 1979; Horowitz, 1991). Of further significance is that these deletions include those sequences encoding the E1b 19- and 55-kD proteins. The 19-kD protein is reported to be involved in inhibiting apoptosis (Rao *et al.*, 1992; White *et al.*, 1992), whereas the 55-kD protein is able to bind wild-type p53 protein (Sarnow *et al.*, 1982; Heuvel *et al.*, 1990). By deleting these adenoviral sequences, we remove potential inhibitors of p53 function through direct binding to p53 or potential inhibition of p53-mediated apoptosis. We have created additional constructs that have had the remaining 3′ E1b sequence, including all protein IX coding sequence, deleted as well. Although this has been reported to reduce the packaging size capacity of adenovirus to approximately 3 kb, less than wild-type virus (Ghosh-Choudhury *et al.*, 1987), these constructs are also deleted in the E3 region so that the A/M/N/53 and A/C/N/53 constructs are well within this size range. By deleting the pIX region, adenoviral sequences homologous to those contained in 293 cells are reduced to approximately 300 bp, decreasing the chances of regenerating replication-competent, wild-type adenovirus through recombination. Constructs lacking pIX coding sequence appear to have equal efficacy and drive equivalent levels of p53 protein expression as those with pIX (unpublished observations).

p53/Adenovirus efficacy in vitro

In concordance with a strong dose dependency for expression of p53 protein in infected cells, we have also demonstrated a dose-dependent, p53-specific inhibition of tumor cell growth by our recombinants. We were able to inhibit cell division, demonstrated by the inhibition of DNA synthesis, in a wide variety of tumor cell types known to lack wild-type p53 protein expression. Bacchetti and Graham (1993) recently reported p53-specific inhibition of DNA synthesis in the ovarian carcinoma cell line SKOV-3 by a p53 recombinant adenovirus in similar experiments. In addition to ovarian carcinoma, we have demonstrated that additional human tumor cell lines, representative of clinically important human cancers and including lines overexpressing mutant p53 protein, can also be growth inhibited by our p53 recombinants. At moi values where the A/C/N/53 recombinant is 90–100% effective in inhibiting DNA synthesis in these tumor types, control adenovirus-mediated suppression is less than 20%.

Although Feinstein *et al.* (1992) reported that reintroduction of wild-type p53 could induce differentiation and increase the proportion of cells in G_1 *versus* S + G_2 for leukemic K562 cells, we found no p53-specific effect in this line. Horvath and Weber (1988) have reported that human peripheral blood lymphocytes are highly nonpermissive to adenovirus infection. In separate experiments, we found that we were not able to infect the nonresponding K562 cells significantly with recombinant A/C/β-Gal adenovirus, while other cell lines, including the control Hep G2 line and those showing a strong p53 effect, were readily infectable (Harris *et al.*, in preparation). Thus, at least part of the variability of efficacy would appear to be due to variability of infection, although other factors may be involved as well. For example, Chen *et al.* (1991) reported that wild-type p53 can suppress tumorigenicity without inhibiting the growth rate of some tumor lines. Alternatively, mutations of regulatory proteins acting downstream from p53 may also exist in some tumor cell lines, limiting the effect of p53 treatment. The lack of a p53-specific effect in the wild-type control cell line Hep G2 is encouraging, suggesting that overexpression of wild-type p53 over endogenous background levels may have only minor effects in normal cells infected with the recombinant.

The ability to treat human cancer cells *ex vivo* and suppress their growth *in vivo* when implanted into an animal is an important step toward identifying promising gene therapy candidates. The results observed with the A/M/N/53 virus in Fig. 5 demonstrates that complete suppression is possible in an *in vivo* environment. The resumption of tumor growth in 2 out of the 4 p53-treated animals at the lower moi most likely resulted from a small percentage of cells not initially infected with the p53 recombinant at this dose. We did not analyze the resulting tumors for the presence of adenoviral genomes. The complete suppression seen with A/M/N/53 at the highr dose, however, shows that the ability of tumor growth to recover can be overcome.

p53/Adenovirus in vivo efficacy

Work presented here and by other groups (Chen *et al.*, 1990; Takahashi *et al.*, 1992) have shown that human tumor cells lacking expression of wild-type p53 can be treated *ex vivo* with p53 and result in suppression of tumor growth when the treated cells are transferred into an animal model. This report presents the first evidence of tumor suppressor gene therapy of an *in vivo* established tumor, resulting in both suppression of tumor growth and increased survival time. Delivery to tumor cells did not rely on direct injection into the tumor mass. Rather, p53 recombinant adenovirus was injected into the peritumoral space, and p53 mRNA expression was detected within the tumor. p53 expressed by the recombinants was functional and strongly suppressed tumor growth as compared to that of control, non-p53-expressing adenovirus-treated tumors. However, both p53 and control virus-treated tumor groups showed tumor suppression as compared to buffer-treated controls. It has been demonstrated that local expression of tumor necrosis factor (TNF), interferon-γ (IFN-γ), interleukin (IL)-2, IL-4, or IL-7 can lead to T-cell-independent transient tumor suppression in nude mice (Hoch *et al.*, 1992). Exposure of monocytes to adenovirus results in the release of TNF, and adenovirus virions are also weak inducers of IFN-α/β (for review, see Gooding and Wold, 1990). Therefore, it is not surprising that we observed some tumor suppression in nude mice even with the control adenovirus. We did not observe this virus-mediated tumor suppression in the *ex vivo* control virus-treated Saos-2 tumor cells described earlier. The p53-specific *in vivo* tumor suppression was dramatically demonstrated by continued monitoring of the animals in Fig. 7. The survival time of the p53-treated mice was significantly increased, with 5 out of 5 animals still alive more than 135 days after tumor cell inoculation compared to 0 out of 5 adenovirus control-treated animals. Two out of 5 mice continue to survive beyond day 170, more than twice the survival time of the longest-lived control virus and buffer-treated animals. The surviving animals still exhibit growing tumors, which may reflect cells not initially infected with the p53 recombinant adenovirus. Higher or more frequent dosing schedules may address this. In addition, promoter shutoff (Palmer *et al.*, 1991) or additional mutations may have rendered these cells resistant to the p53 recombinant adenovirus treatment.

Implications for gene therapy

There will be over one million new cases of cancer diagnosed this year, and half that number of cancer-related deaths (American Cancer Society, 1993). p53 mutations are the most common genetic alteration asociated with human cancers, occurring in 50–60% of human cancers (Bartek *et al.*, 1991; Hollstein *et al.*, 1991; Levine, 1993). The goal of gene therapy in treating p53-deficient tumors is to reinstate a normal, functional copy of the wild-type p53 gene so that control of cellular proliferation is restored. p53 plays a central role in cell cycle progression, arresting growth so that repair or apoptosis can occur in response to DNA damage. The possibility of using p53/adenovirus to drive tumor cells into the apoptotic pathway is intriguing. Wild-type p53 has recently been identified as a necessary component for apoptosis induced by irradiation or treatment with some chemotherapeutic agents (Lowe *et al.*, 1993a,b). Due to the high prevalence of p53 mutations in human tumors, it is possible that tumors which have become refractory to chemotherapy and irradiation treatments may have become so due in part to the lack of wild-type p53. By resupplying functional p53 to these tumors, it is possible that they will now become susceptible to apoptosis normally associated with the DNA damage induced by radiation and chemotherapy.

One of the critical points in successful human tumor suppressor gene therapy is the ability to affect a significant fraction of the cancer cells. Toward that goal, recombinant adenoviruses have distinct advantages over other gene delivery methods (for review, see Siegfried, 1993). Adenoviruses have never been shown to induce tumors in humans and have been safely used as live vaccines (Straus, 1984). Replication-deficient recombinant adenoviruses can be produced by replacing the E1 region necessary for replication with the target gene. Adenovirus does not integrate into the human genome as a normal consequence of infection, thereby greatly reducing the risk of insertional mutagenesis possible with retrovirus or AAV vectors. This lack of stable integration also leads to an additional safety feature in that the transferred gene effect will be transient, as the extrachromasomal DNA will be gradually lost with continued division of normal cells. Stable, high-titer recombinant adenovirus can be produced at levels not achievable with retrovirus or AAV, allowing enough material to be produced to treat a large patient population. Others have shown that adenovirus-mediated gene delivery has a strong potential for gene therapy for diseases such as cystic fibrosis (Rosenfeld *et al.*, 1992; Rich *et al.*, 1993) and α_1-antitrypsin deficiency (Lemarchand *et al.*, 1992). Although other alternatives for gene delivery, such as cationic liposome–DNA complexes, are also currently being explored, none as yet appear as effective as adenovirus-mediated gene delivery.

Here, we have shown that recombinant adenoviruses expressing wild-type p53 can efficiently inhibit DNA synthesis and suppress the growth of a broad range of human tumor cell types, including clinically relevant targets. Furthermore, we have shown that the recombinant adenoviruses can express p53 in an *in vivo* established tumor without relying on direct injection into the tumor or prior *ex vivo* treatment of the cancer cells. The p53 expressed is functional and effectively suppressed tumor growth *in vivo* and significantly increased survival time in a nude mouse model of human lung cancer. Although further studies are needed to ensure the safety of this method of gene delivery and address possible problems of immune responses, the data presented here strongly support the concept of adenovirus-mediated p53 gene therapy of p53-deficient tumors in humans.

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REFERENCES

AMERICAN CANCER SOCIETY. (1993). Cancer Facts and Figures.

BACCHETTI, S., and GRAHAM, F. (1993). Inhibition of cell proliferation by an adenovirus vector expressing the human wild type p53 protein. Int. J. Oncol. **3**, 781–788.

BAKER S.J., MARKOWITZ, S., FEARON, E.R., WILLSON, J.K.V., and VOGELSTEIN, B. (1990). Suppression of human colorectal carcinoma cell growth by wild-type p53. Science **249**, 912–915.

BARTEK, J., BARTKOVA, J., VOJTESEK, B., STASKOVA, Z., LUKAS, J., REJTHAR, A., KOVARIK, J., MIDGLEY, C.A., GANNON, J.V., and LANE, D.P. (1991). Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies. Oncogene **6**, 1699–1703.

BRESSAC, B., GALVIN, K.M., LIANG, T.J., ISSELBACHER, K.J., WANDS, J.R., and OZTURK, M. (1990). Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA **87**, 1973–1977.

CHALLBERG, M.D., and KELLY, T.J. (1979). Adenovirus DNA replication in vitro. Biochemistry **76**, 655–659.

CHEN P.L., CHEN Y., BOOKSTEIN R., and LEE W.H. (1990). Genetic mechanisms of tumor suppression by the human p53 gene. Science **250**, 1576–1580.

CHEN, Y., CHEN, P.L., ARNAIZ, N., GOODRICH, D., and LEE, W.H. (1991). Expression of wild-type p53 in human A673 cells suppresses tumorigenicity but not growth rate. Oncogene **6**, 1799–1805.

CHENG, J., YEE, J.K., YEARGIN, J., FRIEDMANN, T., and HAAS, M. (1992). Suppression of acute lymphoblastic leukemia by the human wild-type p53 gene. Cancer Res. **52**, 222–226.

DILLER, L., KASSEL, J., NELSON, C.E., GRYKA, M.A., LITWAK, G., GEBHARDT, M., BRESSAC, B., OZTURK, M., BAKER, S.J., VOGELSTEIN, B., and FRIEND, S.H. (1990). p53 functions as a cell cycle control protein in osteosarcomas. Mol. Cell. Biol. **10**, 5772–5781.

FEINSTEIN, E., GALE, R.P., REED, J., and CANAANI, E. (1992). Expression of the normal p53 gene induces differentiation of K562 cells. Oncogene **7**, 1853–1857.

GHOSH-CHOUDHURY, G., HAJ-AHMAD, Y., and GRAHAM, F.L. (1987). Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full length genomes. EMBO J. **6**, 1733–1739.

GOODING, L.R., and WOLD, W.S.M. (1990). Molecular mechanisms by which adenoviruses counteract antiviral immune defenses. Crit. Rev. Immunol. **10**, 53–71.

GRAHAM, F.L., and VAN DER ERB, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**, 456–467.

GRAHAM, F.L., SMILEY, J., RUSSELL, W.C., and NAIRN, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**, 59–74.

GRAHAM, F.L., and PREVEC, L. (1991). Manipulation of adenovirus vectors. In *Methods in Molecular Biology*, vol. 7. *Gene Transfer and Expression Protocols*. E.J. Murray, ed. (The Humana Press Inc., Clifton NJ) pp. 109–128.

HEUVEL, S.J.L., LAAR, T., KAST, W.M., MELIEF, C.J.M., ZANTEMA, A., and VAN DER EB, A.J. (1990). Association between the cellular p53 and the adenovirus 5 E1B-55kd proteins reduces the oncogenicity of Ad-transformed cells. EMBO J. **9**, 2621–2629.

HOCK, H., DORSCH, M., KUZENDORF, U., QIN, Z., DIAMANTSTEIN, T., and BLANKENSTEIN, T. (1992). Mechanisms of rejection induced by tumor cell-targeted gene transfer of interleukin 2, interleukin 4, interleukin 7, tumor necrosis factor, or interferon γ. Proc. Natl. Acad. Sci. USA **90**, 2774–2778.

HOLLSTEIN, M., SIDRANSKY, D., VOGELSTEIN, B., and HARRIS C. (1991). p53 mutations in human cancers. Science **253**, 49–53.

HOROWITZ, M.S. (1991). Adenoviridae and their replication. In *Fields Virology*. B.N. Fields, ed. (Raven Press, New York) pp. 1679–1721.

HORVATH, J., and WEBER, J.M. (1988). Nonpermissivity of human peripheral blood lymphocytes to adenovirus type 2 infection. J. Virol. **62**, 341–345.

HSU, I.C., TOKIWA, T., BENNETT, W., METCALF, R.A., WELSH, J.A., SUN, T., and HARRIS, C.C. (1993). p53 gene mutation and integrated hepatitus B viral DNA sequences in human liver cancer cell lines. Carcinogenesis **14**, 987–992.

JONES, N., and SHENK, T. (1979). Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell **17**, 683–689.

KEURBITZ, S.J., PLUNKETT, B.S., WALSH, W.V., and KASTAN, M.B. (1992). Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA **89**, 7491–7495.

LANE, D.P. (1992). p53, guardian of the genome. Nature **358**, 15–16.

LEMARCHAND, P., JAFFE, H.A., DANEL, C., CID, M.C., KLEINMAN, H.K., STRATFORD-PERRICAUDET, L.D., PERRICAUDET, M., PAVIRANI, A., LECOCQ, J.P., and CRYSTAL, R.G. (1992). Adenovirus-mediated transfer of a recombinant human α_1-antitrypsin cDNA to human endothelial cells. Proc. Natl. Acad. Sci. USA **89**, 6482–6486.

LEVINE, A.J. (1993). The tumor suppressor genes. Annu. Rev. Biochem. **62**, 623–651.

LOWE, S.W., RULEY, H.E., JACKS, T., and HOUSMAN, D.E. (1993a). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell **74**, 957–967.

LOWE, S.W., SCHMITT, E.M., SMITH, S.W., OSBORNE, B.A., and JACKS, T. (1993b). p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature **362**, 847–852.

MERCER, W.E., SHIELDS, M.T., AMIN, M., SUAVE, G.J., APPELLA, E., ROMANO, J.W., and ULLRICH, S.J. (1990). Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. Proc. Natl. Acad. Sci. USA **87**, 6166–6170.

PALMER, T.D., ROSMAN, G.J., OSBORNE, W.R., and MILLER, A.D. (1991). Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. Proc. Natl. Acad. Sci. USA **88**, 1330–1334.

RAO, L., DEBBAS, M., SABBATINI, P., HOCKENBERY, D., KORSMEYER, S., and WHITE, E. (1992). The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. Proc. Natl. Acad. Sci. USA **89**, 7742–7746.

RICH, D.P., COUTURE, L.A., CARDOZA, L.M., GUIGGIO, V.M., ARMENTANO, D., ESPINO, P.C., HEHIR, K., WELSH, M.J., SMITH, A.E., and GREGORY, R.J. (1993). Development and analysis of recombinant adenoviruses for gene therapy of cystic fibrosis. Hum. Gene Ther. **4**, 461–476.

ROSENFELD, M.A., YOSHIMURA, K., TRAPNELL, B.C., YONEYAMA, K., ROSENTHAL, E.R., DALEMANS, W., FUKAYAMA, M., BARGON, J., STIER, L.E., STRATFORD-PERRICAUDET, L., PERRICAUDET, M., GUGGINO, W.B., PAVIRANI, A., LECOCQ, J.P., and CRYSTAL, R.G. (1992). In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. Cell **68**, 143–155.

SAMBROOK, J., FRITSCH, E.F., and MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual*. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

SARNOW, P., HO, Y.S., WILLIAMS, J., and LEVINE, A.J. (1982).

Adenovirus E1b-58 kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. Cell **28**, 387–394.

SHAW, P., BOVEY, R., TARDY, S., SAHLI, R., SORDAT, B., and COSTA, J. (1992). Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc. Natl. Acad. Sci. USA **89**, 4495–4499.

SIEGFRIED, W. (1993). Perspectives in gene therapy with recombinant adenoviruses. Exp. Clin. Endocrinol. **101**, 7–11.

STRAUS, S.E. (1984). Adenovirus infections in humans. In *The Adenoviruses*. H.S. Ginsberg, ed. (Plenum Press, New York) pp. 451–496.

TAKAHASHI, T., NAU, M.M., CHIBA, I., BIRRER, M.J., ROSENBERG, R.K., VINOCOUR, M., LEVITT, M., PASS, H., GAZDAR, A.F., and MINNA, J.D. (1989). p53: A frequent target for genetic abnormalities in lung cancer. Science **246**, 491–494.

TAKAHASHI, T., CARBONE, D., TAKAHASHI, T., NAU, M.M., HIDA, T., LINNOILA, I., UEDA, R., and MINNA, J.D. (1992). Wild-type but not mutant p53 suppresses the growth of human lung cancer cells bearing multiple genetic lesions. Cancer Res. **52**, 2340–2343.

THIMMAPPAYA, B., WEINBERGER, C., SCHNEIDER, R.J., and SHENK, T. (1982). Adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times aftr infection. Cell **31**, 543–551.

WANG, A.M., DOYLE, M.V., and MARK, D.F. (1989). Quantitation of mRNA by the polymerase chain reaction. Proc. Natl. Acad. Sci. USA **86**, 9717–9721.

WHITE, E., SABBATINI, P., DEBBAS, M., WOLD, W.S.M., KUSHER, D.I., and GOODING, L.R. (1992). The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor. Mol. Cell. Biol. **12**, 2570–2580.

YONISH-ROUACH, E., RESNITZKY, D., LOTEM, J., SACHS, L., KIMCHI, A., and OREN, M. (1991). Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature **352**, 345–347.

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Section Review

Oncologic, Endocrine & Metabolic

Gene therapy strategies for cancer

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Gene therapy of cancer has undergone an explosive development at its infant stage during the past five years. It is an attractive biotechnology not because it has surpassed conventional cancer therapies but for its potential to provide scientists and clinicians with powerful tools to cure cancer through genetic manipulations. Due to the application of molecular biology techniques to cancer treatment, through intervention in the mechanisms of carcinogenesis, the field of cancer gene therapy is filled with opportunities for innovative design of therapeutic strategies. Based on an overview of the field, this article organises current technologies in cancer gene therapy into six major approaches:

1) Genetic sequence-targeted therapies;
2) Tumour suppressor gene therapy;
3) Toxin or prodrug-activation gene therapy;
4) Drug-resistance gene therapy;
5) Cytokine gene therapy and tumour vaccination;
6) Combinational gene therapy.

On learning about these technologies, each of which may contain several distinct methodologies, it is easy to see that the field of cancer gene therapy is currently in a state of dynamic development.

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Introduction

Cancer is a disease of genes. There is ample evidence that carcinogenesis is a multistage process involving multiple genetic and epigenetic events in proto-oncogenes, tumour suppressor genes, and antimetastasis genes [1,2]. Chemicals, radiation, and viruses all can initiate cellular transformation by attacking genetic material, for example, activating proto-oncogenes or inactivating tumour suppressor genes. The initiated cells, promoted by epigenetic factors and further genetic alterations, can expand themselves and their defects: dysregulated terminal differentiation, lost control of growth, and acquired resistance to cytotoxic effects. This expansion leads to preneoplastic lesions, which progress further through the process of epigenetic influence and genetic disorder and finally reach the stage of clinical cancer.

Gene therapy of cancer is, therefore, a rational strategy for cancer treatment. The potential effectiveness of gene therapy is promised not only by its precise targeting at the mechanisms of the disease, but also by its genetic approaches which are based on rapidly advancing molecular biotechnology.

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Abbreviations

Ad: adenovirus
AML: acute myeloblastic leukaemia
APC: antigen-presenting cell
APC: adenomatosis polyposis coli tumour suppressor gene
araATP: adenine arabinonucleoside triphosphate
araM: 6-methoxypurine arabinonucleoside
BRCA1: breast and ovarian cancer susceptibility gene
cdk: cyclin-dependent kinase
CFTR: cystic fibrosis transmembrane conductance regulator
Cip1: cyclin-dependent kinase-interacting protein 1
CML: chronic myelogenous leukaemia
CTL: cytotoxic T-lymphocyte(s)
DCC: deleted in colon cancer tumour suppressor gene
G-CSF: granulocyte colony-stimulating factor
GCV: ganciclovir
GM-CSF: granulocyte-macrophage colony-stimulating factor
GPAT: genetic prodrug-activation therapy
GSH: L-g-glutamyl-L-cysteinglycine
GST: glutathione-*s*-transferase
HPV: human papilloma virus
HSV-*tk*: herpes simplex virus-thymidine kinase gene
IFN: interferon
IL: interleukin
LAK: lymphokine-activated killer cell
LLC: Lewis lung carcinoma
MDR: multidrug resistance
MDR: multidrug resistance gene
MHC: major histocompatibility complex
MnSOD: manganese superoxide
MTS: major tumour suppressor
NDP: nucleotide diphosphate
Neo^R: neomycin-resistant gene
NF1: neurofibromatosis tumour suppressor gene
PBL: peripheral blood lymphocyte(s)
PFU: plaque-forming unit
PKC: protein kinase C
Rb: retinoblastoma susceptibility gene
TCR: T-cell receptor
TIL: tumour-infiltrating lymphocytes
TNF-α: tumour necrosis factor-α
Topo II: topoisomerase II
VDEPT: virus-directed enzyme/prodrug therapy
VZV-tk: varicella-zoster virus thymidine kinase gene
WAF1: wild-type p53-activated fragment 1
WT1: Wilms tumour suppressor gene

Of all of the gene therapy clinical protocols thus far approved by the federal regulatory agencies, more than 85% are cancer gene therapy trials, indicating that gene therapy of cancer currently has a particular favour for research, development, and application.

There are several reasons for the explosive development of cancer gene therapy from experimental hypotheses to clinical trials:

- the discovery of the genetic basis of cancer inspired the development of genetic approaches to cope with the disease;
- the availability of gene or antigene manipulation technology which was promoted by successful gene therapy of somatic or metabolic genetic diseases;
- the unsatisfactory status of conventional cancer therapies; and
- the desperation of patients who are willing to try novel therapies in the face of a life-threatening disease.

Diverse strategies and innovative approaches to cancer gene therapy have been developed. Figure 1 depicts the current major methodologies of cancer gene therapy. Despite the great potential and rapid advances in this technology, the development of cancer gene therapy remains at a very early stage. The objective of this review is to present an overview of the current state of the field, which may be used as a reference for understanding the prospects of cancer gene therapy.

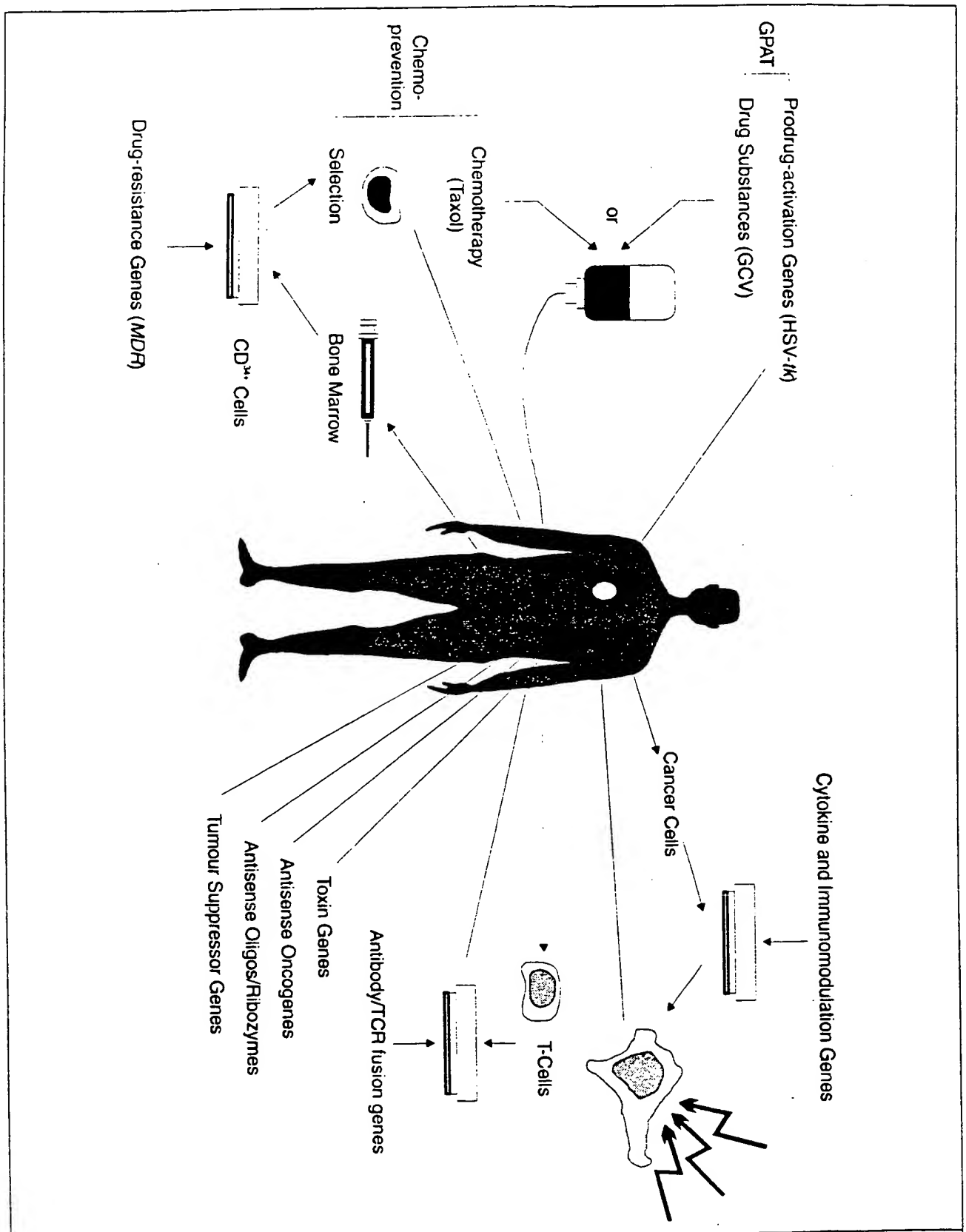

Figure 1: Major approaches of gene therapy for cancer. Shown is a digrammatic summary of the strategies of cancer gene therapy. A hypothetical tumour located in the lung is depicted either as a source for cancer cell isolation or as a target for gene transfer. Each approach is discussed in detail in the text. GPAT, genetic prodrug-activation therapy; HSV-*tk*: herpex simplex virus-thymidine kinase gene; GCV: ganciclovir; *MDR*: multidrug resistance gene; Taxol: paclitaxel; TCR: T-cell receptor; Oligos: oligodeoxynucleotides.

Genetic sequence-targeted therapies

The past five years have witnessed a rapid development of genetic sequence-targeted therapeutic agents. The significant advances in research of gene regulation of cells and molecular mechanisms of diseases have given pharmaceutical scientists a rational basis for the design of drugs that target genetic sequences through specific recognition of and by hydrogen bonding between complementary bases.

Along the pathway for genetic information flow from DNA to protein, several interventions have been developed to modulate gene expression and regulation. Examples include:

- triplex formation of oligonucleotides with double-stranded DNA to block transcription;
- antisense oligonucleotides;
- antisense RNA binding single-stranded DNA or mRNA to interfere with transcription, splicing, and translation;
- ribozymes specifically binding and cleaving target mRNA.

All of these approaches have been demonstrated to be effective in cellular treatments or even in animal models. Their applications for cancer gene therapy are under investigation. Besides these, new approaches are under development, such as oligonucleotides covalently linked to an intercalating agent or to a nucleic acid-cleaving reagent [3], peptide nucleic acids, which possess both antisense and antigene properties [4], and sense oligonucleotide to block transcription factors competitively, or site-specific DNA-binding proteins to block the transcription of oncogenes [5]. On antisense approaches to cancer gene therapy, a recent review published in *Cancer Gene Therapy* provides additional references for and future directions of this type of technology [6].

Antisense oligonucleotides

The first antisense oligonucleotide to be administered to humans, ISIS 2105, is in Phase II clinical tests [7]. The rapid progress in the development of antisense oligonucleotide drugs has been attributed to the synthetic oligonucleotide technology, which allowed creation of a variety of modified oligonucleotides to cope with the rapid degradation of regular oligonucleotides by nucleases *in vivo* [8]. The most promising of the modified ones are phosphorothioate oligonucleotides, which have been shown to be effective against cellular RNA and have attractive pharmacokinetic and toxicological properties in animals [9,10].

Anti-oncogene oligonucleotides have been demonstrated in a variety of cancer cell lines to inhibit effectively the activities of different oncogenes or proto-oncogenes, such as *c-abl*, *c-fos*, *c-fes*, *c-fms*, *c-kit*, *c-myb*, *c-myc*, *c-raf*, *c-src*, and *ras* [9,11,12]. Specific inhibition of p210 mRNA of the *bcr-abl* fusion gene in chronic myelogenous leukaemia (CML) by antisense oligonucleotides is an often cited example [13,14]. Inhibition of gene expression and tumourigenicity by antisense oligonucleotides has also been shown in several tumour or other animal models [15].

In 1992, an antisense oligonucleotide designed to block expression of the *p53* gene was administered systemically to a patient with acute myeloblastic leukaemia (AML) [16]. This was based on the previous observation that p53, though being currently considered as a tumour suppressor, was correlated with the proliferation of AML blast stem cells [17]. No major toxicity was detected from this patient after given a ten-day infusion of the anti-p53 oligonucleotide at a dose of 0.05 mg/kg/hour (total dose: 700 mg). This led to a Phase I dose escalation study with more patients. The 20-mer anti-p53 (exon 10) oligonucleotides had a strong inhibitory effect on the *in vitro* growth and viability of leukaemic blasts from more than thirty patients with AML as compared with medium alone, or with two oligonucleotide controls.

The mechanism by which antisense oligonucleotides inhibit gene expression has not been clearly demonstrated by *in vivo* evidence. The initial thought was that they bound to target mRNA and induced translation arrest [18]. This mechanism may be of importance when the antisense oligonucleotides are targeted to the translation start codon. The oligonucleotides targeting coding sequences of mRNA may induce RNase H to cleave the DNA-bound RNA, inhibiting translation [19]. Several other mechanisms have been postulated:

- the antisense oligonucleotides interfere with transcription by hybridising to single-stranded DNA;
- they inactivate splicing by binding to hnRNA splicing sites;
- they block RNA transportation from nucleus to cytoplasm; and
- they inhibit the initiation translation by binding to ribosomal subunit entry sites.

Precise molecular targeting mechanisms position antisense oligonucleotides as a type of 'informational drug'. Their efficacy in suppressing gene expression has been well established but does not eliminate the pharmacokinetic limitations conferred by the artificial-oligomer nature of the drugs. Lack of delivery speci-

ficity, instability *in vivo*, preferential accumulation in liver and kidneys, low cellular uptake efficiency, and short retention of effective concentrations within cells are the major problems to be resolved for using antisense oligonucleotides systemically or semi-systemically in the clinic. Although the entry of the oligonucleotide into cells has been shown through receptor-mediated endocytosis [20,21] or recently *via* protein kinase C (PKC)-dependent pinocytosis [22], the internalisation pathways cause the drugs to be retained or destroyed in the cellular vesicle system, limiting their ability to reach action sites at an effective concentration. The receptor-mediated pathway also poses the potential problem that drug uptake will be dependent on the availability of the receptor on the cell surface.

Current approaches to improving the *in vivo* stability and delivery of antisense oligonucleotides range from modification of oligonucleotides to construction of oligonucleotide-liposome complexes [23]. Examples include end modification [24], lipophile conjugation [25], polylysine conjugation with liposome encapsulation [26], and linking of antibodies with oligonucleotide-liposome complex [27]. At one time, researchers wondered whether antisense oligonucleotides were the real 'magic bullet', but the evidence collected thus far does not support this [12]. It is feasible that they could become novel antiviral agents, but much more development is needed before they can confidently be called a new type of anticancer agent. Additional information can be obtained in a recent review on this subject [28].

Antisense RNA

Antisense RNAs occur naturally in prokaryotic and eukaryotic cells. They have been shown to play regulatory roles in several cellular processes, including DNA replication, transcription, RNA processing, and translation, all through a base-pairing mechanism [29,30]. Their ability to inhibit gene expression specifically led to the use of artificial antisense RNA to study biological function in both prokaryotic and eukaryotic systems [31,32]. This was soon exploited for the development of new approaches for therapy against viruses and cancer.

Antisense RNAs in their DNA template forms can be constructed in expression cassettes, carried by plasmids or viral vectors, and produced either constitutively or following induction in targeted cells. In this aspect, antisense RNAs have an obvious advantage over antisense oligonucleotides, since their templates can be efficiently delivered into target cells by viral vectors or other means and their active forms can be produced within the cells in a controllable manner by different promoters.

Suppression of transcription or translation of proto-oncogenes or oncogenes by antisense RNA has been successfully demonstrated on c-*fos* [33], c-*myc* [34], and K-*ras* [35], in each case leading to reversion of the transformation phenotypes of the target cells. The K-*ras* experiment used a novel design of the antisense RNA template in which a 2 kb K-*ras* genomic fragment containing the first and second exons with the first intron in between was used for specifically attacking K-*ras* mRNA. This strategy was further utilised for generating an anti-K-*ras* recombinant retrovirus [36]. The virus was shown to have a strong inhibitory effect on the growth and tumourigenicity of human lung cancer cells that have a mutated K-*ras*.

It was also shown greatly to reduce tumour formation in mouse models of orthotopic human lung cancer [37]. These findings led to the development of a clinical trial protocol that has been approved by the NIH for the treatment of unresectable lung cancer by direct intratumour injection of the anti-K-*ras* recombinant retrovirus.

A set of mechanisms for the activity of antisense RNA have been proposed that are very similar to those for antisense oligonucleotides:

- the antisense RNA interferes with transcription;
- it blocks RNA splicing and exportation;
- it inhibits translation; and
- it induces RNase III to cleave double-stranded RNA after binding to target mRNA.

In vivo evidence of these mechanisms has not been found. Double-stranded RNA complexes have rarely been isolated. There are unique problems associated with the antisense RNA approach, such as the formation of self-inhibitory secondary structure, the degradation by nuclease, and the variations in expression level and persistence. Further development of this approach will focus on optimising regulation of antisense RNA expression and producing targeted, efficient delivery for DNA constructs.

Antigene oligonucleotides

The antigene oligonucleotide approach extends from the strategy of antisense oligonucleotides. Antigene oligonucleotides bind to double-stranded DNA through Hoogsteen hydrogen bonding [38], which, distinct from Watson-Crick base-pairing, occurs between polypurine or polypyrimidine oligodeoxynucleotides and double-stranded polypurine or polypyrimidine stretches in DNA. Antigene oligonucleotides occupy the major groove of the DNA helix and specifically suppress the activities of targeted genes. The biological effects of antigene oligonu-

cleotides were first shown by their blocking the access of sequence-specific proteins to the same or neighbouring sequences [39,40]. Homopyrimidine oligonucleotides bound to the homopurine-homopyrimidine sequence inhibited restriction enzyme cleavage and/or methylation by methylases. Binding of a transcription factor, Sp1, was inhibited by a triplex-forming oligonucleotide that overlapped the Sp1 binding site by four base pairs. Specific inhibition of transcription by formation of triplex complexes was demonstrated in the transcription assays of the human c-*myc* gene *in vitro* [41] and the interleukin-2 (IL-2) receptor gene *in vivo* [42].

Two major types of DNA triplexes have been described: intramolecular and intermolecular [43]. In an intermolecular triplex, a separate third strand associates with a target duplex DNA. In an intramolecular triplex, the third strand is a portion of one of the strands of the duplex that has folded back to associate with the purine-pyrimidine tract. The triplex DNA structures have been confirmed by x-ray crystallography, nuclear magnetic resonance, ultraviolet absorption spectroscopy, circular dichroism, enzymatic probing, and chemical probing [43].

The antigene strategy requires that the target sequence be accessible within the chromatin structure in the nucleus. It was shown that the oligonucleotide can reach the nucleus of cells that are incubated with a micromolar concentration of the oligonucleotide [44], but a large part of the oligonucleotide remained trapped in endocytic vesicles and was not available to induce biological effects. Efforts to improve cellular uptake of antigene oligonucleotides have been similar to those used for antisense oligonucleotides. To increase stability, nuclease-resistant oligonucleotides, such as oligo-[α]-deoxynucleotides, which can be synthesised with the α-anomers of nucleotide units, have been applied [45]. Strategies for improving the effectiveness of antigene oligonucleotides include:

- modification of oligonucleotides with unnatural residues [43];
- linkage of oligonucleotides with intercalating or cross-linking agents; and
- conjugation with reactive groups such as cleaving reagents [46].

The antigene oligonucleotide approach is in the early development stage. It has constraints similar to those of antisense oligonucleotides, e.g., instability, lack of delivery specificity, low efficiency of cellular uptake, and inability to sustain effective concentrations in target cells. Besides, its activity is limited to the availability of polypurine stretches in the targeted gene. If these weaknesses can be overcome, the antigene oligonucleotide strategy is potentially useful for cancer gene therapy.

Ribozymes

Ribozymes are RNA molecules that possess specific catalytic activities [47]. Among different types of ribozymes [48,49], the best characterised group for the purpose of gene therapy is the hammerhead ribozymes. The consensus hammerhead model consists of three base-paired stems, I, II, and III, and a core region in which thirteen residues are strictly conserved for cleaving immediately 3′ to any GUX sequence (where X is C, A, or U).

In the design and construction of a hammerhead ribozyme, specific sequences can be introduced in stems I and III that impose complementary recognition between the ribozyme and target RNA. Hammerhead ribozymes can perform a true enzymatic reaction, during which a substrate is cleaved and the ribozyme itself is not altered, thereby enabling multiple reaction cycles.

The utility of hammerhead ribozymes specifically designed for inhibiting oncogene activities has been demonstrated by anti-*ras* ribozymes [50,51]. In one study, a ribozyme was specifically designed to cleave the H-*ras* mRNA at the mutated codon 12 in human bladder carcinoma EJ cells. The ribozyme, encoded by a synthetic DNA, was carried by a eukaryotic expression vector and transfected into EJ cells. The expressed ribozyme significantly altered the morphology and suppressed the growth of the EJ cells *in vitro*. Reductions in the expression of H-*ras* mRNA and p21 protein in the EJ cells were shown in reciprocal by the increase in expression of the ribozyme RNA. The tumourigenic potential of the EJ cells was greatly inhibited by the ribozyme in a mouse model. Another study used a set of similar ribozymes also targeted at the mutated codon 12 of the H-*ras* mRNA. Plasmids containing the ribozyme-encoding sequences were stably transfected in NIHT3 cells, which were then transfected with the activated H-*ras* gene. The ribozymes were found to inhibit transformation of NIH3T3 cells by the H-*ras* oncogene. Both of the studies suggested that anti-oncogene ribozymes may be developed as a new class of anticancer agents.

Like other RNA molecules, exogenous ribozymes in target cells suffer from rapid degradation by nucleases, which can directly affect the concentration of the ribozymes and subsequent cleavage efficacy. Attempts have been made to increase the stability of the ribozymes by modifying their primary structure, for example, by adding an extra sequence at the 3′ end of

the molecules [52]. Slow turnover of ribozymes, a result of strong binding between the substrate RNA and ribozymes, is another drawback that affects their efficiency as enzymatic drugs, making them behave more like antisense RNA in cells. Targeted delivery of ribozyme constructs to cancer cells *in vivo* remains to be developed. A retroviral vector has been used to carry a ribozyme sequence in the development of an anti-HIV agent [53]. The use of adeno-associated virus vectors to carry ribozyme constructs specific for binding and cleaving the E6 and E7 transcripts of human papillomavirus has been reported [54], and efficient cleavage of the cognate targets *in vitro* were demonstrated under a variety of conditions, including at physiological temperature. Further research and development of ribozyme-mediated *in vivo* cleavage of target transcripts of oncogenes or HIV may lead to clinical trials of this type of approach.

Tumour suppressor gene therapy

The genetic bases of cancer include abnormalities in oncogenes and/or tumour suppressor genes. Both types have been the targets of cancer gene therapy. Because the cancer-related defects of tumour suppressor genes are usually mutations or deletions, the strategy in tumour suppressor gene therapy thus far developed has been gene replacement therapy: a wild-type tumour suppressor gene is transferred into cancer cells to restore the normal function of the defective gene. Although this approach appears to be straightforward and logical, it does have several technical hurdles to overcome:

- crucial defective gene(s) must be identified for a given type of cancer in order to reverse the malignant phenotype or induce a tumouricidal effect;
- a highly efficient and targeted delivery system is required;
- the therapeutic gene needs to be delivered into enough target cells to elicit a bystander effect; and
- expression of the therapeutic gene must be controllable and not harmful to normal cells.

Moreover, taking into account the fact that cancer is not a monogenic disease, and often comprises multiple lesions in different oncogenes and tumour suppressor genes, it is reasonable to assume that replacement of a single gene will not always be sufficient to reverse the malignant phenotype. However, the current single-gene replacement therapy has provided encouraging data that support further development of this approach.

Tumour suppressor genes

The fusion of normal and malignant tumour cells has led to the suppression of tumourigenicity in many different combinations. This phenomenon provided the first evidence that the normal genome might contain 'recessive cancer genes' [55]. Following the identification and cloning of the retinoblastoma susceptibility (*Rb*) and *p53* genes, the field has progressed exponentially. Although tumours develope through multiple changes in several genes, a malignant phenotype has been reversed by the introduction of a single chromosome derived from a normal cell, suggesting that a single suppressor gene may be able to overcome the effects of multiple tumour progression-related cytogenetic changes [56]. The human tumour suppressor genes that have been cloned and characterised include *Rb*, Wilms tumour (*WT1*), and neurofibromatosis (*NF1*), which are involved in paediatric cancers; adenomatosis polyposis coli (*APC*) and deleted in colon cancer (*DCC*), which contribute to colorectal cancer; and *p53*, which is found in mutated forms in a wide range of human cancers. More recently, the development of animal models using methods to knock out tumour suppressor genes has demonstrated that disruption of either the *Rb* or *p53* gene yields mice that are prone to cancer formation [57]. Understanding these mechanisms has provided the basis for direct gene replacement therapy, by which abnormal tumour suppressor genes can be corrected.

Retinoblastoma susceptibility (Rb) gene

The *Rb* gene has been shown to be inactivated in virtually all retinoblastomas as well as in a number of adult tumours, such as small cell lung cancer and cancers of the breast, prostate, and bladder. The *Rb* gene product, in its unphosphorylated form, appears capable of blocking the cell cycle in the G1 phase, thereby maintaining cells in a quiescent state. *Rb* is inactivated by phosphorylation; the cell is allowed to proceed to DNA replication and mitosis [58]. Although genetic events other than *Rb* abnormalities may initiate malignant transformation, loss of the Rb protein can remove a residual checkpoint of cell-cycle control, thereby further promoting the growth and/or progress of cancer cells.

Introduction of the normal *Rb* gene into retinoblastoma or osteosarcoma cells with inactivated endogenous *Rb* genes affected cell morphology, growth rate, soft agar colony formation, and tumourigenicity in *nu/nu* mice [59]. A similar activity has been shown in human prostate carcinoma cell lines. These findings suggest that the normal *Rb*-encoded protein may be used clinically for adult neoplasms [60].

p53 gene

Named the 'molecule of the year' in 1993 by the journal *Science*, the *p53* tumour suppressor gene has been at the centre stage of cancer research, attracting the attention of biologists and clinicians. Of all the known genes with therapeutic potential in cancer, *p53* is the most extensively studied [61,62]. Mutations to the *p53* gene and allele loss on chromosome 17p, where this gene is located, are among the most frequent alterations yet identified in human malignancies. The p53 protein is highly conserved through evolution and is expressed in most normal tissues. Wild-type p53 has been shown to be involved in control of the cell cycle [63,64], transcriptional regulation [65,66], DNA replication [67,68], and induction of apoptosis [69-71]. The wild-type *p53* gene can suppress cell transformation and neoplastic cell growth [72-75]. Overexpression of the *p53* gene product is associated with mutations in *p53* and significantly correlates with a poor prognosis [76,77]. In addition to somatic mutations, it has also been reported that germ-line *p53* mutations were associated with Li-Fraumeni syndrome, a familial cancer disease [78]. In studying the mechanism by which the p53 protein plays a role in cell-cycle control, a *p53*-regulated 21 kDa protein was identified as cyclin-dependent kinase-interacting protein 1 (Cip1) or wild-type p53-activated fragment 1 (WAF1) by two independent groups [79,80]. These studies not only revealed a G1 cyclin-dependent kinase control by p53 through p21, but also connected the p53 function to that of Rb through p21 and G1 cyclin-dependent kinases. The mechanisms of action of the p53 tumour suppressor and prospects for cancer gene therapy by reconstitution of p53 function were discussed in a recent review [81].

Other tumour suppressor or cancer susceptibility genes

The *DCC* and *nm23* genes are also candidates for gene replacement therapy, for colon cancer and metastatic cancers, respectively. The *DCC* gene product has sequence homology to fibronectin-like cell adhesion molecules. The deletion of this gene, which is involved in the transmission of signals generated by cell interactions, is thought to be a relatively early event in the development of colon cancer [82]. The *nm23* gene is considered to be a metastasis suppressor gene and to encode nucleotide diphosphate (NDP) kinase. Some types of tumour cells transfected with this gene lost their metastatic potential when applied in animal models [83,84]. Recently, two major events occurred in the area of identification of new tumour suppressor genes or cancer susceptibility genes. First, two highly related members of the cyclin-dependent kinase (cdk) inhibitor family, termed *p16* (major tumour suppressor 1, *MTS1*) and *p15* (*MTS2*), were isolated from the chromosomal region 9p21 [85,86]. Second, a strong candidate for the breast and ovarian cancer susceptibility gene *BRCA 1* was identified [87]. While *p16* was shown to be deleted or mutated in a wide range of cancer cell lines, *p15* was shown to be a potential effector of TGF-β-induced cell cycle arrest [88]. Despite some challenges to the *p16* gene as a major tumour suppressor [89], further studies demonstrated that alteration or deletion of *p16* and *p15* did occur in different types of primary tumours at a lower frequency than that in tumour cell lines [90-93]. Applications of these new genes in cancer therapy will depend on further research.

Tumour suppressor gene therapy

The germ cells of patients with Li-Fraumeni syndrome are heterozygous for wild-type and mutant *p53*, whereas the tumours of these patients are homozygous for mutant *p53*. Mice that have a homozygous deleted *p53* developed a variety of tumours as early as six months of age, whereas, the heterozygous mice that contain one wild-type *p53* allele developed tumours at a reduced frequency and at a slower rate [57]. These observations suggest that the reintroduction of a single copy of the wild-type *p53* gene may be able to reverse malignant phenotypes in tumour cells. Several studies demonstrated that expression of the wild-type *p53* gene suppressed proliferation of human tumour cell lines that lack *p53* or express mutant *p53 in vitro* [73-75,94]. *In vivo* experiments in nude mice also showed that tumour cells expressing wild-type *p53* are no longer tumourigenic or are less tumourigenic than parental cells.

The research in this area is progressing rapidly and has demonstrated that the restoration of wild-type p53 function in tumour cells could be a very efficient approach to cancer therapy. The early experiments showed that intratracheal instillation of retrovirus containing wild-type *p53* prevented the growth of established orthotopic human lung cancer in *nu/nu* mice. Further studies, using a multicellular tumour spheroid model, also showed evidence that retroviral vectors were capable of penetrating into three-dimensional structures and that exposure of spheroids to a retroviral vector expressing wild-type *p53* induced programmed cell death in lung cancer cells [95]. These findings suggest that this gene replacement technique may be useful as an adjuvant in eliminating residual cancer cells following surgery and primary radiation of lung cancer. Moreover, this approach is feasible for other tumours such as colon cancer, which is also frequently associated with *p53* mutations. Mutations in *p53* are found in Barrett's epithelium, the premalignant precursor lesion to adenocarcinoma of the oesophagus, and are frequently associated with second primary cancers

of the aerodigestive tract [96,97]. This raises the intriguing possibility that cancer could be prevented by reversing genetic mutations in premalignant lesions with local-regional instillation of viral vectors.

To achieve a more efficient delivery of the *p53* gene into lung cancer cells *in vivo*, a replication-defective and helper-independent recombinant *p53* adenovirus was generated [98]. The virus, Ad5CMV-*p53*, carries an expression cassette that contains human cytomegalovirus E1 promoter, human wild-type *p53* cDNA, and SV40 early polyadenylation signal. Human non-small cell lung cancer cell lines representing different *p53* configurations were used to evaluate the expression of the Ad5CMV-p53 virus. In the H358 cell line, which has a homozygous deletion of *p53*, the *p53* gene was transferred with 95 - 100% efficiency, as detected by immunohistochemical analysis, when the cells were infected with Ad5CMV-*p53* at a multiplicity of infection of thirty to fifty plaque-forming units (PFU)/cell. Western blots showed that the p53 protein was expressed at a high level in these cells. Growth of the lung cancer cells with *p53* deletion or mutation was greatly inhibited by Ad5CMV-*p53*, while that of the cell line containing wild-type *p53* was less affected. Tumourigenicity tests in nude mice demonstrated that Ad5CMV-*p53* prevented tumour formation [99]. Induction of apoptosis was shown to be one of the mechanisms for the tumouricidal effect of Ad5CMV-p53 [100]. The Ad-mediated *p53* gene transfer has also been demonstrated to be effective in suppressing tumourigenicity in different animal models [101,102]. These results suggest that adenovirus is an efficient vector for mediating transfer and expression of tumour suppressor genes in human cancer cells and that the *p53* Ad may be further developed into an effective therapeutic agent in cancer. Clinical trials of Ad-mediated *p53* gene transfer for treatment of lung, head and neck, and liver cancers are underway.

Toxin or prodrug-activation gene therapy

Toxin gene transfer into cancer cells represents a straightforward approach in cancer gene therapy. The therapeutic index will be largely dependent on the specificity of gene delivery and efficiency of gene transfer *in vivo* to target tumour cells. Genetic prodrug-activation therapy (GPAT) represents a selective genetic strategy against cancer. This involves delivery of a prodrug activating enzyme gene into both tumour and normal cells. By linking the enzyme gene downstream of tumour-specific transcription units, tumour-specific prodrug activation can be achieved. For a detailed review on the prodrug-activation genes for selective cancer chemotherapy, reference is made to a recent article in *Cancer Gene Therapy* [103].

Toxin gene therapy

Cell killing by expression of the diphtheria toxin A chain coding sequence has been demonstrated *in vitro* [104] and in animals [105,106]. Diphtheria toxin is an extremely potent inhibitor of protein synthesis in eukaryotic cells. It has been estimated that one molecule of diphtheria toxin A per cell is sufficient to kill murine L cells [107]. The toxin is composed of two subunits. The B chain (342 amino acids) is adsorbed to the cell surface for internalisation and the A chain (193 amino acids) specifically modifies histidine residues of elongation factor 2 by ADP-ribosylation, which prevents protein synthesis and kills the cell. Expression of the toxin may be induced by linking the diphtheria toxin A coding sequence with tissue-specific transcription regulatory elements (promoters and enhancers). This approach has been tested in B-lymphoid cells, in which a plasmid was transfected that contained the diphtheria toxin A chain coding sequence under control of the engineered immunoglobulin kappa light chain gene regulatory sequences [108]. This construct specifically expressed diphtheria toxin A in mature B-cells but not in pre-B-cells, suggesting that the construct may be further developed to allow therapeutic ablation of malignant B-cells of mature stages while sparing normal progenitor cells.

A similar method used *Pseudomonas* exotoxin, which was conjugated with IL-4 to treat murine sarcoma and colon adenocarcinoma cells that express high-affinity IL-4 receptors [109]. The chimeric IL-4 *Pseudomonas* exotoxin protein was shown to be cytotoxic to the tumour cells by inhibiting cellular protein synthesis in a dose-dependent manner. A nonchimeric *Pseudomonas* exotoxin protein that could not bind to the IL-4 receptor did not inhibit protein synthesis in tumour cells. A chimeric mutant protein that could bind to IL-4 receptor but did not have the capacity to inhibit protein synthesis was not cytotoxic to tumour cells. The protein synthesis-inhibitory activity of the IL-4/*Pseudomonas* exotoxin fusion protein could be completely abolished by a neutralising antibody to IL-4. These data suggest that a receptor-mediated toxin therapy might be effective.

A new approach in the technology of toxin gene therapy is transfer of the E1A gene of the type-5 adenovirus into tumours. The E1A protein has been shown to be able to induce apoptosis [110,111]. This protein was also shown to suppress expression of the *HER-2/neu* proto-oncogene in cancer cells [112]. The frequent amplification or overexpression of the *HER-2/neu* gene, observed in different types of human cancer, has been shown to correlate with shorter survival time or lower survival rate in ovarian cancer patients. Based on these observations, a strategy to use E1A as a therapeutic gene for treatment of ovarian

cancers that overexpress *HER-2/neu* was developed [113].

Prodrug-activation gene therapy

Prodrug-activation gene therapy is based on the introduction of a drug sensitivity gene into target cells, which are then killed by administration of the drug at doses that are not detrimental to normal cells. One such 'prodrug-activation' gene that has been successfully used to confer drug sensitivity in an animal model system is the herpes simplex virus thymidine kinase gene (HSV-*tk*). The HSV-TK enzyme can specifically catalyse the phosphorylation of a number of nucleoside analogues, such as acyclovir or ganciclovir, which are poor substrates for the TK enzymes of mammalian cells [114]. The phosphorylated acyclic nucleoside becomes active when incorporated into newly synthesised DNA, resulting in a cytocidal effect by induction of DNA strand breaks and inhibition of DNA polymerase activity [115].

In a syngeneic mouse model, subcutaneous tumours developed from the HSV-*tk*-gene transduced tumour cells went into complete regression following intraperitoneal administration of ganciclovir, while the tumours derived from the nontransduced tumour cells were not affected [116]. An analogous approach has been taken for treatment of brain tumours in a rat model [117]. In these experiments, the HSV-*tk* retrovirus-producing cells were stereotactically injected into rat cerebral gliomas *in vivo*. The HSV-*tk* retroviruses generated from the producer cells were expected to infect the proliferating tumour cells preferentially, which would then be killed selectively by ganciclovir administered intraperitoneally. Indeed, complete regression of the glioma was observed in eleven of fourteen rats [117]. Since it was unlikely that all of the tumour cells became infected with the HSV-*tk* retroviruses, it was suggested that this regression may have been due to a 'bystander effect'. This effect was demonstrated by a co-culture experiment *in vitro*, in which HSV-*tk* transduced human fibrosarcoma cells induced the ganciclovir-killing effect on nontransduced co-culture cells through a gap junction-mediated metabolic co-operation [118]. Although the mechanism of the bystander effect *in vivo* was not well understood, the remarkable success of this technique in the treatment of a very aggressive tumour that has an extremely poor prognosis when treated with conventional therapy has led to human clinical trials [119].

The therapeutic approach called 'virus-directed enzyme/prodrug therapy' (VDEPT) is another example of prodrug-activation gene therapy [120]. In treatment of hepatocellular carcinoma with VDEPT, the varicella-zoster virus thymidine kinase (VZV-*tk*) gene, that was transcriptionally regulated by either the hepatoma-associated alpha-fetoprotein or liver-associated albumin promoters, was constructed into a retroviral vector. After infecting the cancer cells with this vector, nontoxic prodrug 6-methoxypurine arabinonucleoside (araM) activated by VZV-*tk* was expressed preferentially in hepatoma cells. The final product, adenine arabinonucleoside triphosphate (araATP), selectively induced cytotoxicity in the hepatoma cells that expressed the gene.

The gene encoding cytosine deaminase can also be used to prime cell death upon administration of a drug that is not normally toxic to eukaryotic cells [121]. Cytosine deaminase converts the nontoxic substance 5-fluorocytosine to a toxic derivative, 5-fluorouracil. Thus, only genetically modified cells carrying and expressing the cytosine deaminase gene are able to synthesise 5-fluorouracil and induce the cytocidal effect. Retrovirus-mediated cytosine deaminase gene transfer in various cell types has demonstrated this specific cell killing effect after treatment of the transduced cells with 5-fluorocytosine [122].

Recent advances in this technology are improvements in enhancing tumour-killing efficacy and reducing side-effects on normal cells by tissue- or cell-specific expression of prodrug-activation genes. Several recent publications describe in detail the designs and applications of this approach [123-125]. Also, experimental tumour therapy in mice using cyclophosphamide-activating cytochrome P450 2B1 gene transfer into glioma models has been reported to sensitise the tumour cells to the cytotoxic effects of cyclophosphamide [126]. It was suggested that the *in situ* activation of cyclophosphamide by cytochrome P450 2B1 may provide a novel approach for brain tumour gene therapy.

Using radiation to sensitise tumour cells preferentially for gene therapy is a new approach [127]. Transcriptional regulation of the promoter/enhancer region of the *Egr-1* gene can be specifically activated by ionising radiation. A plasmid construct, made by linking the promoter region of *Egr-1* to tumour necrosis factor (TNF)-α, was stably transfected into a human haematopoietic cell line, which exhibited 3.2-fold induction of TNF-α upon radiation at 20 Gy. The radiosensitising *Egr*-TNF cells were injected into human xenografts of the radioresistant squamous cell carcinoma cell line SQ-20B in nude mice. Animals treated with the *Egr*-TNF cells and radiation demonstrated an increase in tumour cures compared with animals treated with radiation alone or unirradiated animals given injections of the cells alone [127].

Drug-resistance gene therapy

This approach, also called chemoprotection, adopts a strategy that is the opposite of toxin or prodrug-activation gene therapy. It protects drug-sensitive cells, such as bone marrow stem cells, from chemotherapeutic drugs with a drug-resistant gene, which allows the sensitive cells to survive treatment with chemotherapeutic drugs so that a maximal tumouricidal effect can be achieved. A recent review article provides supplemental references for gene transfer of drug resistance genes and implications for cancer therapy [128].

Multidrug resistance gene and other chemoresistant genes

Chemoresistance is one of the principal obstacles to effective systemic treatment of cancer [129]. The high frequency of a phenomenon called multidrug resistance (MDR), seen both in the clinical treatment of cancer and in tissue culture models, suggests that cancer cells may have specific gene products that confer simultaneous resistance to many different kinds of anticancer drugs. When highly resistant cell clones were selected by sequential multiple drugs [130], a gene, named as *MDR1*, was isolated, and the level of 4.5 kb mRNA in cells correlates with the degree of resistance [131]. The *MDR1* gene was soon shown to encode a 170 kDa membrane glycoprotein known as P-glycoprotein [132]. The predicted structure of the protein includes two groups of six transmembrane domains, with each group containing an ATP binding/utilisation site within a cytoplasmic region. This identifies the P-glycoprotein as a member of the transporter superfamily [133], which also includes the CFTR protein, a sex peptide transport system from yeast, and bacterial nutrient and polypeptide toxin transport systems.

Morphological, biochemical, and physiological studies have shown that the product of the *MDR1* gene is an ATP-dependent multidrug transporter [134]. Three independent lines of investigation have provided evidence that P-glycoprotein is responsible for MDR in many instances:

- the *MDR1* gene is overexpressed in a number of MDR cell lines [132,135];
- transfer and expression of the *MDR1* gene is sufficient to induce MDR in drug-sensitive cells [136,137];
- expression of the human *MDR1* gene in transgenic mice has been shown to result in resistance to drug-induced bone marrow suppression [138].

Identification and characterisation of *MDR1* and its product P-glycoprotein were the key steps that led to understanding of the mechanism of MDR. However, experimental observations have revealed that MDR is a heterologous cellular response to cytotoxic drugs, suggesting that the mechanism of MDR is more complex than may be hypothesised on the basis of the single P-glycoprotein [139]. There are multiresistant cell lines that do not express the P-glycoprotein, indicating that other drug-resistant mechanisms may exist [140]. Examples of proteins that confer drug resistance by some other mechanisms include protein kinases, glutathione-*S*-transferase, and topoisomerase II.

An increase in the level of protein kinase C (PKC) was observed in the MDR cells of human mammary carcinoma. Treatment with phorbol esters, which activate PKC, increases resistance and reduces the intracellular accumulation of doxorubicin [141]. Analogous results have been shown with AMP-dependent protein kinase A activity. Phosphoprotein changes accompanying the development of resistance to mitomycin C were detected in human colon tumour cells [142].

Glutathione-*S*-transferase (GST) catalyses the coupling reaction of intracellular electrophiles with L-g-glutamyl-L-cysteinglycine (GSH). The multifunctional isozymes of GST, which are found in virtually all tissues, are involved mainly in phase II of liver detoxification, in which xenobiotics are inactivated by GST through conjugation. Elevation of GST has been shown to be associated with the acquired resistance of cells to certain anticancer drugs [143]. Further studies showed that the elevated expression of GST correlated with increased resistance to alkylating agents [144,145].

The protein topoisomerase II (Topo II), also known as DNA gyrase, is responsible for the cytotoxicity of anthracycline, acridine, and etoposide, which exert their cytotoxic effect through the formation of a stable ternary complex of DNA-Topo II-drug that alters the processes of DNA duplication and transcription [146]. Different drugs have been shown to alter Topo II activities in different types of cancer [140]. In resistant leukaemia cell lines, the catalytic activity of Topo II was three to five times lower than that of the sensitive parental lines [147].

Chemoprotection

Multidrug resistance is a major obstacle in cancer chemotherapy. Great research and clinical efforts have been applied to circumvent MDR activities since the MDR genes and their molecular mechanisms were discovered [148]. The two most effective approaches thus far developed are combining chemotherapy either with chemosensitisers that enhance the sensitivity of

target cells to drugs or with immunotherapy that suppresses MDR protein activities. The consideration of MDR as a gene therapy application led to a third, very innovative approach, called chemoprotection. This approach exploits the MDR genes selectively to protect drug-sensitive normal tissues such as bone marrow. After transfer of one of these genes, dosages of anticancer drugs can be raised high enough to kill cancer cells by overcoming their drug resistance.

This approach originated from work in the *MDR1* transgenic mouse model [138]. The high levels of *MDR1* gene products expressed in the transgenic mice successfully protected the sensitive bone marrow from cytotoxic drugs. This effect was confirmed by transplantation of bone marrow from the transgenic *MDR1* mice to drug-sensitive mice, which conferred drug resistance on the recipient animal [149]. These results suggested that it might be possible to introduce the *MDR1* gene into bone marrow *ex vivo*, making that bone marrow resistant to many drugs, and return it to the patient to increase tolerance of and response to high-dose chemotherapy. That expression of the transferred *MDR1* gene is stable and selective in mouse bone marrow has recently been demonstrated by two groups [150,151]. The *MDR1* gene was also shown to function as a dominant selectable marker *in vivo*, allowing a minority of genetically engineered bone marrow cells to be positively selected and enriched by the administration of cytotoxic drugs such as paclitaxel (Taxol) [151]. Chemoprotection from Taxol was evaluated in mice transplanted with *MDR1*-modified bone marrow cells [152]. These mice showed resistance at doses of Taxol that were lethal to mice not transplanted with the *MDR1*-modified marrow. Taxol-resistant haematopoiesis was sustained through five consecutive transplants with *MDR1*-modified marrow, showing that sufficient numbers of early progenitor cells were modified and repopulated under selection by Taxol. Similar Taxol-resistant effects were also obtained with transplantation of human *MDR1* cDNA-modified marrow cells into mice [153]. The success of chemoprotection in the animal models has led to the design of a clinical trial of chemoprotection against Taxol in advanced-stage human epithelial cancers, such as ovarian cancer, by using a similar approach of *MDR1*-modified autologous bone marrow transplantation.

The dihydrofolate reductase gene was similarly used in a retroviral transfer into murine recipients to protect from methotrexate-induced cytopenia. The study demonstrated that mice transplanted with bone marrow cells infected with a retroviral dihydrofolate reductase expression vector showed improved protection from methotrexate-induced marrow toxicity and longer survival than control mice; however, enrichment of transduced cells by *in vivo* selection could not be detected [154].

A new approach in treatment of cancer with protection of normal tissues is radioprotection. Overexpression of manganese superoxide (MnSOD) has been postulated as one possible mechanism of radioprotection for haematopoietic cells [155]. In this study, the human *MnSOD* gene constructs in both the sense and antisense orientation were transduced into K562 and A375 cells by retroviral vectors. Results demonstrated that K562 cells transduced with *MnSOD* in the antisense orientation displayed increased sensitivity to irradiation and that, in contrast, A375 cells transduced with the sense *MnSOD* gene displayed increased resistance to irradiation compared to both parental or vector-transduced cells. It was proposed that administration of genetically engineered haematopoietic stem cells transduced with *MnSOD* alone or in combination with other antioxidant enzymes could allow for the purging of tumour cells, using higher-dose irradiation *ex vivo*, and may improve the resistance of bone marrow cells during high-dose radio- or chemotherapy *in vivo* [155].

Cytokine gene therapy and tumour vaccination

Major progress has been made in the understanding not only of the molecular mechanisms underlying carcinogenesis, but also of the complex relations between cancer and the immune system. Among all of the approaches of gene therapy for cancer, cytokine gene therapy and tumour vaccination have a unique strategy that aims at utilising host immune responses to suppress or eliminate tumour cells, which is particularly important for metastatic cancers. Transfer of some cytokine or costimulatory factor genes has been demonstrated to induce immune responses protecting the animal against subsequent injection of parental tumour cells, and can even, in some cases, treat efficiently animals carrying pre-existing parental tumours. The rapid advances in this methodology formed the basis for the recent elaboration of a fast increasing number of clinical trials using cytokine gene transfer for treatment of cancer.

Genetic modulation of lymphocytes

It was the genetic modification of lymphocytes by retrovirus-mediated gene transfer that initiated human gene therapy [156,157]. The reasons for choosing lymphocytes as gene-transfer recipients were:

- lymphocytes are readily isolated from patients and cultured *in vitro*;

- tumour-infiltrating lymphocytes were identified as a specific antitumor immunoeffector and their kinetics *in vivo* needed to be studied; and
- ethical and safety considerations required that retrovirus-mediated gene transfer first be tested through an *ex vivo* approach.

The clinical trials in which lymphocytes were used to carry a marker or therapeutic gene actually started the *ex vivo* gene transfer technology that is now very useful in various cancer gene therapies, such as gene-modified autologous tumour cell vaccination.

Gene marking in tumour-infiltrating lymphocytes

Tumour-infiltrating lymphocytes (TIL) are lymphoid cells that accumulate in tumour masses. TIL are isolated from resected tumours by incubation of the heterogeneous single cell suspension derived from tumours with IL-2 [158,159]. Some TIL have the ability to recognise antigens associated with autologous tumour and to kill tumour cells, as well as secrete cytokines [160,161]. In one animal model in which lymphokine-activated killer cells (LAK) are relatively ineffective, TIL were shown to mediate the regression of established tumour deposits with a potency fifty to one hundred times that of LAK cells [158]. In clinical trials, it was possible to grow TIL from approximately 50% of the tumours, and these TIL kill autologous tumour cells from approximately one-third of patients with melanoma [159,162]. Studies of the distribution of TIL following intravenous injection demonstrated that TIL could accumulate in tumour deposits [163,164]. Among more than fifty patients with metastatic melanoma who underwent TIL adoptive therapy, 38% demonstrated an objective regression; however, these responses were of short duration [165]. Both animal and clinical data suggested that TIL may be genetically modified to improve their antitumor therapeutic effectiveness.

Gene marking was used in the first phase of the genetic modification of TIL. The cells were transduced with the neomycin-resistant gene (Neo^R) by retrovirus-mediated gene transfer and introduced into humans for study of their long-term distribution and survival [156,166]. The experimental results showed that the Neo^R gene was well expressed and the modified TIL had no detectable changes in their general properties. After the infusion of the gene-modified TIL into patients, the cells were detectable in the circulation for up to 189 days and in tumour deposits for up to sixty-four days. Safety tests performed on samples from the patients who received the gene-modified cells were all negative and no antibodies against the vector retrovirus were detected by western blot assays of patients' serum at varying times up to 180 days after cell infusion [156]. This was the first clinical trial that used autologous cells as vehicles for retrovirus-mediated gene transfer in man. The publication of this trial had a profound impact on human gene therapy; it provided data that supported the feasibility and safety of using autologous cells as vehicles for retrovirus-mediated gene transfer. Following the example of TIL-gene marking, many other gene marking experiments have been carried out using this *ex vivo* technique. For example, marking autologous marrow with the Neo^R gene was intended to identify whether the cells responsible for relapse were the gene-transferred cells or residual cells in patients [167]. A proposed protocol that would use dual retroviral markers to test the relative contributions of marrow and peripheral blood autologous cells to recovery after reparative therapy for chronic myelogenous leukaemia is expected to be initiated this year [168].

Cytokine gene-modified tumour-infiltrating lymphocytes

As an extension of the Neo^R-TIL gene-marking study, modification of TIL by addition of the gene for tumour necrosis factor (TNF) has been studied in patients with malignant melanoma. Tumour necrosis factor (TNF) produces very encouraging antitumour responses in mice, but the maximal tolerated dose in humans is about forty-fold less than the doses required to elicit these responses in mice [169]. Toxic effects are common at doses above 8 mg/kg in man, whereas in the mouse 400 mg/kg can be achieved. Therefore, a strategy was developed to deliver TNF effectively to the site of the tumour using TIL as cellular vehicles [170]. The TIL were isolated directly from the tumour and then grown in large numbers in tissue culture with IL-2. After expansion in culture, they were genetically engineered to produce TNF and were given to the patient intravenously along with high doses of IL-2 for several days. The results of this clinical trial are not yet available.

It was proposed that TIL could also be modified with other genes such as those that encode interferons (IFN-β or IFN-γ), other cytokines (IL-1α, IL-6, and IL-7), or receptors (of Fc, chimeric T-cells, or IL-2) [171]. However, TIL have several drawbacks for use in cytokine gene transfer:

- technical difficulties in isolation and culture;
- poor efficiency for gene transduction;
- weak expression of the transduced gene; and
- low percentage of homing (< 0.015% per gram of tumour).

These were demonstrated by a recent comparison of TNF gene expression in TIL *versus* the melanoma cell lines that were derived from the same enzymatically

digested tumour biopsies used for isolating TIL [172]. Therefore, an alternative approach is being pursued, using tumour cells as vehicles to carry cytokines and other immune-modulating genes for systemically boosting the antitumor immune response.

Single chain antibody gene-modified T-lymphocytes

Since the isolation, expansion, and tumour-homing efficiency of TIL are limited, an experimental approach which exploits the targeted cytolytic activity of lymphocytes is being developed [173]. Two basic designs, composed of either double chimeric T-cell receptor chains (cTCR) or single-chain Fv linked to the signal transducing γ or ζ subunits of the FcR or CD3 (scFvR), have been constructed. Both chimeric receptor genes when transfected into human leukaemic T-cells, TIL or peripheral blood lymphocytes (PBL), endow the recipient T-cells with non-MHC restricted, antibody-type specificity [174]. Upon binding to antigen (either immobilised or displayed on the surface of target cells), the chimeric receptors could transmit a signal for T-cell activation. Since this approach consists of modifying T-cells with an antibody gene, it is also called 'T-body' technology.

In this approach, several technical aspects need to be considered:

- Consistent alterations of tumour cell surface antigens are available as targets;
- The genes of the monoclonal antibodies (mAb) against the antigens are cloned;
- A chimeric gene needs to be constructed that encodes a fusion protein of the Fv region of mAb, a linkage fragment, and the transmembrane and cytoplasmic region of the T-cell receptor (TCR)/CD3 (e.g., ζ chain);
- A high-efficient vector to deliver the chimeric gene into T-cells is necessary.

A recent report on this type of technology described generation of CTLs with specificity for ERBB2 receptor-expressing tumour cells [175]. Overexpression of the ERBB2 receptor is frequently observed in human breast and ovarian carcinomas and provides a target at the cell surface which strongly distinguishes tumour cells from their normal counter parts. A binding function was conferred directly on the ζ chain of the TCR complex to circumvent major histocompatibility complex-restricted antigen recognition through the α and β chains of the TCR. A chimeric gene was constructed which encoded a single-chain Fv antibody (scFv, consisting of the joined heavy- and light-chain variable domains of a monoclonal antibody against the extracellular domain of the ERBB2 receptor), a hinge region as a spacer, and the ζ chain of the TCR. This gene was introduced into CTLs by retroviral gene transfer. The signalling potential of the scFv/hinge/ζ receptors was demonstrated by secretion of interferon γ upon coincubation with ERBB2-expressing cells. Target cells expressing the ERBB2 gene were lysed *in vitro* with high specificity by the scFv/hinge/ζ-expressing T-cells. The growth of ERBB2-transformed cells in athymic nude mice was retarded by adoptively transferred scFv/hinge/ζ-expressing CTLs. Transduced CTLs labelled with a fluorescent dye were specifically detected in tumour sections. These results suggest that tumour cell lysis by CTLs grafted *in vitro* with major histocompatibility complex-independent recognition could become a gene-therapy approach to cancer treatment.

Cytokine gene-modified tumour cell vaccination

The use of autologous tumour cells as vaccines to augment antitumor immunity has been explored throughout this century [176]. Although only a few tumour-specific surface antigens, which can be recognised by effector killer cells such as T-cells, have been identified, local cytokine production is believed to play an important role in invoking tumour immunity [177-179]. Studies have shown that transfer of a cytokine gene into tumour cells leads to continuous local delivery of the cytokine. In these studies, local secretion of cytokines by gene-modified tumour cells not only stopped tumour growth but also, in certain murine models, induced a specific immunity to subsequent tumour challenge. In some cases, immunisation with gene-modified tumour vaccines resulted in regression of existing parental tumours. With the evidence obtained from the individual cytokine studies described herein, a firm basis can be laid for proposing that production of new tumour vaccines by gene transfer or genetic modulation will be a powerful tool for enhancing antitumour immunity. It is also feasible that combinations of these cytokines in genetic modification of tumour vaccines will be even more effective, representing one of the most promising approaches in the development of cancer gene therapy. For further references on experimental and clinical studies of cytokine gene-modified tumour cells, please refer to a recent detailed review in *Human Gene Therapy* [180].

Interleukin-4

Interleukin-4 (IL-4) is produced by the Th2 subset of activated T-helper cells as well as by mast cells. IL-4 participates in the regulation of growth and differentiation of B-cells and T-cells and the generation of cytotoxic T-lymphocytes (CTL). It also activates microvascular endothelium and induces upregulation of vascular cell adhesion molecule I, which potentiates extravasation of lymphocytes and monocytes from the

circulation [181]. Because of these potent immunostimulatory effects, IL-4 may be an ideal cytokine to use for cancer gene therapy alone or in combination with other cytokines.

The therapeutic effects of a cytokine-transduced tumour vaccine were first demonstrated in murine models: murine IL-4-producing plasmacytomas were shown to induce a local antitumour inflammatory response characterised by infiltrates of macrophages and eosinophils [182]. When murine renal tumour cells were engineered to secrete large doses of murine IL-4 locally, the animal hosts rejected these modified tumours and developed systemic immunity against the parental tumour, which was primarily mediated by $CD8^+$ T-cells [183].

Using murine IL-4 retroviral vectors, murine fibroblasts and tumour cells were transduced to produce from 50 - 5000 U of IL-4/10^6 cells/twenty-four hours as determined by ELISA and bioassay. In blinded studies using C57BL/6 and BALB/c mice, it was shown that tumours were significantly inhibited (mean delay of ten days) or in some cases completely suppressed by the co-injection of viable tumour cells with IL-4-producing fibroblasts [184]. Animals that were able to reject an initial tumour inocule could also completely reject subsequent parental tumour challenge of 10^5 cells, while challenge of 10^6 parental tumour cells resulted in a significant delay of tumour induction. In addition, co-administration of systemic IL-2 led to enhancement of *IL-4* gene therapy resulting in a twenty-day delay of pre-established tumour growth compared with controls. A Phase I clinical trial of *IL-4* gene modified antitumor vaccines is in progress.

Interleukin-2

Interleukin-2 (IL-2), first described as a T-cell growth factor, is a cytokine with highly pleiotropic activity [185]. It is an activator of LAK cells [186] and is necessary for activation of TIL cytotoxicity [158]. Many groups have analysed the tumourigenicity and immunogenicity of tumours engineered to produce IL-2. Expression of high levels of IL-2 by the gene-modified tumour cells resulted in regression of even large inocula of tumours. Histologic evaluation of the regressed IL-2 expressing tumours revealed a massive infiltrate of lymphocytic cells [187-189]. Recent studies have indicated that locally activated LAK are a critical local effector in rejection of *IL-2*-transfected tumours. Vaccination with *IL-2* gene-modified tumour cells inhibited experimental metastases in syngeneic immune-competent mice [190]. For this kind of therapy, IL-2 production by tumour cells at the very site of the antigen-MHC may be crucial for efficient processing and presentation of tumour antigen; this was suggested by a recent study in which the use of mammary stromal fibroblasts as vehicles of local IL-2 delivery failed to protect against mouse mammary tumour cells in mice [191].

The expression of the *IL-2* gene in human tumour cell lines was further studied using retroviral and adenoviral vectors [192,193]. The successful retrovirus-mediated transduction of the *IL-2* gene into human melanoma and renal cancer cells and the secretion of IL-2 protein from these tumour cells even after *in vitro* gamma-irradiation has been reported [192,194]. To understand better the functional differences in the anti-tumour responses of immune and tumour-bearing mice, an *in vitro* model to analyse interactions between splenic lymphocytes and tumour cells was used. It was shown that spleen cells isolated from tumour-bearing mice remained unresponsive, while those from immune mice proliferated well in response to IL-2-secreting tumour cells. Only spleen cells from immune animals were able to develop cytotoxicity against tumour cells following *in vitro* restimulation [195]. Clinical trials immunising with autologous or allogeneic tumour cells modified by transduction of the *IL-2* gene are currently in progress. Supplemental references on IL-2 for tumour vaccination can be obtained from other review articles [196,197].

Interferon gamma

Interferon gamma (IFN-γ) is an activator of macrophages and plays an important role in inflammatory responses. This pleiotropic cytokine is also a potent inducer of MHC class I and class II antigens and, thus, is capable of enhancing immune responses [198,199].

Retroviral transduction of cDNA coding for murine IFN-γ into a nonimmunogenic murine sarcoma cell line that expresses MHC class I antigen only weakly induced upregulation of the class I antigen expression and generated $CD8^+$ TIL, which were therapeutic against parental tumour cells. After tumour rejection, long-lasting protection from rechallenge with parental cells was induced [200]. Moreover, immunisation of mice which had micrometastases with tumour cells producing large amounts of IFN-γ almost completely cured these mice by inducing CTL [201].

The cDNA for human IFN-γ was also introduced into human renal cancer cells and melanoma cells [192,194]. Renal cancer cells secreting IFN-γ showed increased expression of MHC class I antigen, β2-microglobulin, and intracellular adhesion molecule I, as well as induction of MHC class II antigen expression. However, tumour formation by a human renal cancer cell line transplanted into *nu/nu* mice was not affected by IFN-γ secretion, whereas IL-2 production inhibited the tumour growth.

Further experimental data on *IFN*-γ-mediated tumour vaccination demonstrated that transduction of human melanoma cells with *IFN*-γ gene enhanced cellular immunity [202]. The *IFN*-γ-transduced and corresponding parental melanoma cells were used for the induction of short-term lymphocyte cultures. Peripheral blood lymphocytes or lymph node cells from twenty melanoma patients were stimulated for five to fifteen days with autologous or MHC class I-matched allogeneic parental or *IFN*-γ-transduced melanoma cells. Seven of the twenty lymphocyte cultures showed substantial increases in lytic activity following stimulation with the transduced melanoma cells in comparison to control lymphocyte cultures stimulated with unmodified parental melanoma. The cytolytic activity stimulated with *IFN*-γ-modified melanomas was mediated partly by MHC-restricted cytotoxic T-lymphocytes and partly by NK cells. Lymphocyte cultures that displayed increases in cytotoxicity after stimulation with the *IFN*-γ-transduced melanoma cells also exhibited enhanced expression or induction of one or more of the following lymphokines: IL-4, IL-1α, IL-1β, IFN-γ, and TNF-α. These studies led to a clinical trial of human *IFN*-γ-transduced autologous tumour cells in patients with disseminated malignant melanoma.

Tumour necrosis factor alpha

Tumour necrosis factor alpha (TNF-α) is a potent immunomodulatory molecule affecting the function of many cells involved in the immune response and tumour vasculature, including T-cells, B-cells, neutrophils, monocytes, and macrophages. TNF-α induces MHC class I and class II molecules [203]. Tumour necrosis factor (TNF) was originally described as an antitumor agent *in vivo*, and it has been demonstrated that injection of recombinant TNF-α can mediate the necrosis and regression of a variety of established murine tumours [204,205]. However, the biological effects of TNF *in vivo* depend on its concentration. At an optimal concentration, TNF produces beneficial effects such as cytotoxic and antitumor effects, while higher concentrations of TNF induce harmful effects such as catastrophic tissue injury, organ failure, and irreversible shock leading to death [206]. Although the exact mechanisms of the *in vivo* effects of TNF-α are not known, it appears to have a significant enhancement on $CD8^+$ T-cells.

The *TNF*-α gene can be successfully introduced into murine and human tumour cells by retroviral vectors. Weakly immunogenic tumour cells modified by the *TNF*-α gene grew slowly *in vitro* and were found to regress after an initial phase of growth. This tumour regression was abrogated by depletion of $CD4^+$ and $CD8^+$ subsets *in vivo*, suggesting that these T-cells are involved in the immune response [207]. In a separate study, macrophages were observed at the sites of murine plasmacytomas that were engineered to secrete TNF-α, implicating inflammatory cells in the rejection of this tumour [208]. However, nonimmunogenic murine fibrosarcoma cells that produced TNF grew progressively in syngeneic mice, although IL-2 was effective in inhibiting this growth [209]. Clinical trials involving immunisation of melanoma patients with autologous TNF-producing tumour cells are in progress.

Interleukin-7

Interleukin-7 (IL-7) was initially described as a growth factor for B-cell progenitors; however, this protein has been demonstrated to stimulate the growth of mature $CD4^+$ and $CD8^+$ T-cells, inducing LAK activity [210]. After stimulating T-cells *via* an antibody against T-cell receptor, an IL-7-dependent, IL-2-independent proliferative pathway has been identified, suggesting that IL-7 may function in the absence of IL-2 to regulate T-cell proliferation. Moreover, IL-7 is capable of inducing tumouricidal activity by peripheral blood monocytes [211].

Murine plasmacytoma cells producing IL-7 were completely rejected after injection into mice. The immune response was primarily mediated by $CD4^+$ T-cells [212]. However, an *IL-7*-transduced murine fibrosarcoma was heavily infiltrated with $CD8^+$ T-cells that were believed to be responsible for the slow growth and tumour regression [213]. Antitumour CTL, generated by drainage of lymph node cells and culture with IL-7, are effective in treating three-day pulmonary metastases of syngeneic methylcholanthrene-induced sarcoma in murine models. Furthermore, systemic administration of IL-2 was synergistic with IL-7-expanded cells in this model, but IL-7 was not. Interleukin-7 is the only cytokine other than IL-2 that has been shown to expand therapeutically effective lymphocytes when adoptively transferred to tumour-bearing mice [214]. Clinical trials on *IL-7* gene therapy for patients with metastatic colon cancer, renal cell cancer, malignant melanoma or lymphoma are ongoing.

Granulocyte-macrophage colony-stimulating factor

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a potent factor that is most often associated with the growth and differentiation of haematopoietic progenitors. Several reports suggest that GM-CSF plays an important role in the maturation and/or function of specialised antigen presenting cells [215]. Incubation of GM-CSF in mouse marrow cultures generated large numbers of dendritic cells, which are potent antigen-presenting cells [216].

In a recent study, vaccination of mice with irradiated tumour cells engineered to secrete murine GM-CSF

was demonstrated to stimulate potent, specific, and long-lasting antitumour immunity [217]. When this murine GM-CSF tumour vaccination model was compared to other cytokine-transduced tumours by the same retroviral vector, it was shown that GM-CSF was the most potent stimulator of systemic antitumor immunity among the ten proteins tested, including IL-2, IL-4, IL-5, IL-6, GM-CSF, IFN-γ, IL-1RA, (ICAM), (CD2), and TNF-α [217]. The success of immunisation with *GM-CSF*-transduced tumours was dependent on both $CD4^+$ and $CD8^+$ T-cells, despite the fact that the tumours were MHC class II negative. The potency of GM-CSF's effect locally might relate to its unique ability to promote the differentiation of haematopoietic precursors to dendritic cells, which could specifically enhance tumour-antigen presentation. Clinical trials of *GM-CSF*-gene mediated tumour vaccination for prostate and renal cell cancers have begun.

Granulocyte colony-stimulating factor

Granulocyte colony-stimulating factor (G-CSF) is characterised as a potent differentiation-inducing factor, essentially in granulopoiesis [218]. Murine colon adenocarcinoma cells expressing G-CSF were rejected from syngeneic mice or *nu/nu* mice. Histologic examinations demonstrated that the antitumour effect was associated with a massive infiltration of neutrophilic granulocytes [219,220]. In contrast, G-CSF tumour vaccines were unable to eradicate an already established G-CSF-secreting carcinoma without the aid of T-lymphocytes [221].

The results of studies that compared the cytokine genes in different tumour cells suggested that the effect of immunomodulation by tumour engineering might depend on both the responsiveness of host cell types and the immunogenicity of tumour cells. Although the results varied in the different tumour systems, because of differences in cell dose, type of cytokine, expression level of transduced gene, and location of immunisation, the effectiveness of autologous tumour vaccines has been well established. The success of the cytokine gene-modified tumour vaccines is believed to depend on a paracrine mechanism in which a locally high concentration of cytokines induces an antitumour immune response, which can be either specific or non-specific. High concentrations of cytokines often induce a non-specific local inflammatory response, which causes the injected tumour cells to be eliminated and immunisation to fail. An alternative in autologous tumour vaccine strategy is to modulate tumour cell antigen presentation by transferring genes such as MHC, B7, and others. This approach may avoid the cytokine-induced local inflammatory response while still inducing local immunisation.

Antigen presentation gene modulation for tumour cell vaccination

Many tumour cells have been shown to carry tumour antigens or tumour-associated peptides that should be capable of activating host T-cells. However, secondary cell-surface changes as well as effects of immunosuppressive factors may render them defective in antigen presentation and inadequate in activation of tumour-specific T-lymphocytes. Several studies in which tumour-specific T-lymphocyte activity was amplified have shown that such amplification can result in potent tumour-specific immunity that can mediate tumour rejection in the autologous host [187,222,223]. Experimental studies have defined a key role for T-lymphocytes in rejection of these tumours, and both $CD4^+$ and $CD8^+$ T-cells have been implicated in tumour rejection [224]. Activation of $CD4^+$ T-cells is thought to require two signals from the antigen-presenting cell (APC). The first signal is the engagement of the T-cell receptor (TCR) for the antigen by the MHC II/peptide complex of the APC [225]. The second signal is thought to be the interaction of the B7 co-stimulatory molecules of the APC with its cognate receptors, CD28 and/or CTLA-4, on the responding T-cell [226-229]. The delivery of the two signals has been shown to be linked and to be connected with the engagement of the class II/peptide complex by the TCR-initiated intracellular signalling events within the APC that stimulated upregulation of B7 on the APC surface [230].

It has long been known that MHC molecules, both class I and class II, are involved in tumour antigen presentation, but through different pathways. Class I MHC can enhance activation of tumour-specific CTL *in vitro*. Early work on tumour vaccination that used transfection of MHC class I genes resulted in suppression of the tumour cells in tumourigenicity and/or metastasis in mouse models [231,232]. MHC class II genes were shown to be involved in activation of tumour-specific T-helper cells, and their introduction into tumour cells resulted in a decrease in the tumourigenicity and generated a systemic immune response against the parental tumour [222]. Despite these positive results, the relationship between levels of MHC expression and immunogenicity is inconsistent among tumour models. Researchers have recently begun to believe that the inconsistency is caused by other cofactors, such as the B7 co-stimulatory molecule, which affects the antigen presentation by MHC/peptide complexes.

The molecule B7 is normally expressed as an activation antigen on antigen-presenting cells such as B-cells, macrophages, and dendritic cells. There are two distinct B7 molecules: B7-1 is the ligand for CD28 and CTLA4, and B7-2 is an alternative ligand for CTLA4 [233,234]. Although the function of CTLA-4 (expressed

exclusively on $CD8^+$ T-cells) is as yet unknown, much evidence has accumulated that CD28 (expressed on all $CD4^+$ and most $CD8^+$ T-cells) is a critical receptor for co-stimulatory signals in T-cell activation. Since CD28 has been shown not only to enhance the level of lymphokine production by $CD4^+$ T-cells subsequent to T-cell receptor engagement but also to promote engagement of $CD8^+$ cells for CTL priming or activation, it is rational to select the B7 gene for transfer into tumour cells to enhance their immunogenicity.

A number of laboratories have reported that, after transfection with a B7 gene, autologous tumours were rejected in their syngeneic host. In addition, systemic immune responses were induced which abrogated the parental tumours at a distant site [235-238]. B7 showed different potency in different types of tumours and animal models. Expression of the co-stimulatory ligand B7 on melanoma cells was found to induce the rejection of a murine melanoma *in vivo*. This rejection was mediated by $CD8^+$ T-cells; $CD4^+$ T-cells were not required. [236]. When mouse Sal sarcoma cells that bore a truncated MHC II molecule and were transfected with B7 cDNA were injected into the mice, they became resistant to challenges of wild-type MHC II^-B7^- ascites or solid Sal tumour; the induced immunity required $CD4^+$ T-cells and was specific for the immunising sarcoma cells [237]. In another case, the transduction of B7 into a tumour by itself was insufficient to cause rejection or systemic immune response; it was necessary to introduce an additional 'strong' tumour antigen into the tumours to obtain the B7-enhanced tumour vaccine effect [238]. In this study, the transfection of B7 into MHC I^+/II^- tumour cells did not enhance their immunogenicity, but the co-introduction of the B7 and MHC II genes did. Although application of B7 to modify tumours is not the final answer to tumour vaccine design, these studies have opened up a novel approach to construction of artificial tumour antigens and their presentation to T-cells as part of the generation of a systemic antitumour immune response.

Combinational gene therapy

This type of methodology seeks to achieve increased efficacy of treatment through the additive or synergistic effects of two or more approaches of cancer gene therapy or gene therapy with conventional cancer therapy. The early report on the strategy of combinational therapy was of tumour suppressor gene therapy combined with chemotherapy. The p53 protein is an inducer of apoptosis, as is the chemotherapeutic agent Cisplatin. When tumours of animal models were treated locally with Ad5CMV-*p53* and systemically with Cisplatin, a stronger tumouricidal effect with local massive apoptosis of tumour cells was detected [239]. The possible synergistic mechanism underlying this effect may be that the DNA damage caused by Cisplatin in tumour cells that were infected with Ad5CMV-*p53* enhanced the proportion of cells entering the p53-dependent apoptotic pathway [240]. This approach has been incorporated into the clinical trial of Ad5CMV-*p53* in lung cancer gene therapy, which will be carried out in 1995 at the University of Texas MD Anderson Cancer Center.

Another strategy for combinational therapy is to express antisense RNA to block the E6 and E7 oncoproteins of human papilloma virus (HPV) and sense RNA for supplementation of tumour suppressor gene *Rb* in cervical cancer cells [241]. The HPV E6 and E7 proteins bind to and inactivate the products of the *p53* and *Rb* genes, which contribute to the carcinogenesis of the cervix and are responsible for the neoplastic phenotype of cervical carcinoma. Co-transfection of the plasmids that carry the E6 and E7 genes in antisense orientation and the *Rb* cDNA in sense orientation into HeLa cells inhibited the cell growth *in vitro* and suppressed tumourigenicity in mouse subcutaneous model [241]. This strategy was designed to reverse the process of HPV-mediated cervical carcinogenesis through suppressing expression of the E6 and E7 genes and restoring the function of the *Rb* gene for cell growth control. The efficacy of this approach in cervical cancer treatment needs to be evaluated further.

In attempts to develop more effective tumour vaccines, transfer of two or more cytokine genes into tumour cells may be an option. Gene therapy experiments have been performed on gene therapy for Lewis lung carcinoma (LLC) [242]. *TNF* and *IL-2* cDNAs were introduced into pBMG-Neo and pcDV-x819 vectors, respectively, and then cotransfected into LLC cells. The co-transfectants were selected by incubating them in a medium containing G418 followed by limiting dilution to obtain *IL2* and *TNF* co-transfected LLC (LLC-TNF-IL-2) cells. When 5×10^5/ml LLC-TNF-IL-2 cells were incubated for forty-eight hours, they secreted 7.56 U/ml TNF and 527.0 U/ml IL-2 into the culture supernatant. When C57BL/6 mice were transplanted with 1×10^6 LLC-TNF-IL-2 cells, all the tumours were rejected. The growth of transplanted LLC, but not B16F10 melanoma cells, was retarded in mice inoculated with LLC-TNF-IL-2 on their contralateral sides, which suggested specific immunity was induced. The immunisation effect by the co-transfectant was superior to that of the *IL-2*- and *TNF*-transfectants alone.

Recently, there have been publications on combinational gene therapies in which mouse cancer models were treated with local cell killing by HSV-*tk*/ganci-

clovir (HSV-*tk*/GCV) system plus induction of anti-tumour immunity by virus-mediated co-expression of IL-2 or GM-CSF [243,244]. In these experiments, suppression of tumourigenicity by using combinational treatment was significantly stronger than using either approach alone. In addition, systemic anti-tumour immune responses were developed in this combinational gene therapy, which was effective against challenges of tumourigenic doses of parental tumour cells inoculated at distant sites. These results suggest that combination of prodrug-activation and cytokine gene therapies *in vivo* can be a powerful approach for treatment of metastatic cancers.

Combinational interventions among different approaches of gene therapies or through gene therapy with conventional therapies are at an early stage. The rationale for the use of combinational therapy is primarily to improve the effectiveness of a single method that is currently not sufficient to fulfill the therapeutic purpose. Generally, before any specific approach of cancer gene therapy with high efficacy is well developed, combinational therapies will be a useful application in cancer treatment.

Future prospects

Dynamic development with diversified technical approaches is the current status of the field of cancer gene therapy; this makes its definition rather difficult. Generally, cancer gene therapy uses genetic modulation of cancer cells or anticancer immunity to suppress malignancy through targeted delivery of therapeutic or regulatory genes or genetic therapeutic agents. The diversity of the field indicates that there may be many ways to achieve an anticancer effect through different genetic manipulations. Nevertheless, it also reflects that there is as yet, no consensus on a dominant approach for any given condition. Every approach yet tried has its own strengths and weaknesses. The field is still very young but does have a bright future.

Several technical challenges now require focused attention with intensive research effort if development of cancer gene therapy is to be accelerated. These include:

- The development of better *in vivo* delivery systems with higher therapeutic indexes, focusing on larger gene-carrying capacity, targeted gene delivery, and more efficient gene transfer with less immunogenicity and low cytotoxicity.
- The controllable transgene expression in target cells: on/off switch, sustained, or inducible tissue-specific expression, etc.
- The comparative study of tumour vaccines, which will clarify the relative potency and specificity of cytokines, allowing construction of combined tumour vaccines with specificity to different tumour types.
- The enhancement of tumour antigen presentation by modulating tumour cells with multiple genes such as MHC, B7, and those for specific tumour targeting.
- The development of novel genetic approaches to the prevention of cancer.

We now realise that no single approach to gene therapy will suffice; rather, we need to develop several complementary strategies that can be combined in various ways to treat a given genetic disease effectively. This is particularly true in cancer gene therapy, since cancer is a disease developed through a multistep process of multiple cytogenetic abnormalities. Gene therapy is unlikely to replace conventional cancer therapy, but rather provides an alternative approach. In the near future, combinational approaches among gene therapies or gene therapy with conventional therapies such as chemotherapy, immunotherapy, radiotherapy, and surgery will be widely used, perhaps leading to the development of a more advanced biological therapy for cancer.

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References

Papers of special interest have been highlighted as:
- • of interest
- •• of considerable interest

1. HARRIS CC: **Molecular basis of multistage carcinogenesis. In:** *Multistage Carcinogenesis*. Harris CC, Hirohashi S, Ito N, Pitot HC, Sugimura T, Terada M, Yokota J (Eds.). Japan Sci. Soc. Press, Tokyo/CRC Press, Boca Raton (1992):3-19.

2. WEINBERG RA: **Oncogenes, tumour suppressor genes, and cell transformation: trying to put it all together. In:** *Origins of Human Cancer: A Comprehensive Review*. Brugge J, Curran T, Harlow ED, and McCormick F (Eds.). Cold Spring Harbor Laboratory Press, New York (1991):1-16.

3. • STEIN CA, COHEN JS: **Oligodeoxynucleotides as inhibitors of gene expression: a review.** *Cancer Res.* (1988) 48:2659-2668.

A detailed review of oligodeoxynucleotides as inhibitors of gene expression.

4. HANVEY JC, PEFFER NJ, BISI JE, THOMPSON SA, CADILLA R, JOSEY JA, RICCA DJ, HASSMAN CF, BONHAM MA, AU KG, CARTER SG, BRUCKENSTEIN DA, BOYD AL, NOBLE

SA, BABISS LE: **Antisense and antigene properties of peptide nucleic acids.** *Science* (1992) **258**:1481-1485.

5. CHOO Y, SANCHEZ-GARCIA I, KLUNG A: ***In vivo* repression by a site-specific DNA-binding protein designed against an oncogenic sequence.** *Nature* (1994) **372**:642-645.

6. MERCOLA D, COHEN JS: **Antisense approaches to cancer gene therapy.** *Cancer Gene Ther.* (1995) **2**:47-59.
• A recent review on antisense approaches to cancer gene therapy.

7. CROOKE ST: **Oligonucleotide therapeutics: a prospectus.** *Antisense Res. Develop.* (1993) **3**:1-2.

8. WICKSTROM E: **Oligodeoxynucleotide stability in subcellular extracts and culture media.** *J. Biochem. Biophys. Methods* (1986) **13**:97-102.

9. HELENE C: **Rational design of sequence-specific oncogene inhibitors based on antisense and antigene oligonucleotides.** *Eur. J. Cancer* (1991) **27**:1466-1471.
• A good review about design of sequence-specific oncogene inhibitors based on antisense and antigene oligonucleotides.

10. CROOKE RM: ***In vitro* toxicology and pharmacokinetics of antisense oligonucleotides.** *Anti-Cancer Drug Design* (1991) **6**:609-646.

11. PROCHOWNIK EV: **Antisense approaches to assessing oncogene signaling pathway. In:** *Gene Regulation: Biology of Antisense RNA and DNA*. Erickson RP, Izant JG (Eds.). Raven Press Ltd., New York (1992) **1**:303-316.

12. STEIN CA, CHENG YC: **Antisense oligonucleotides as therapeutic agents - is the bullet really magical?** *Science* (1993) **261**:1004-1012.
• A good discussion on the basic science and application of oligos as therapeutic agents.

13. SZCZYLIK C, SKORSKI T, NICOLAIDES NC, MANZELLA L, MALAGUARNERA L, VENTURELLI D, GEWIRTZ AM, CALABRETTA B: **Selective inhibition of leukemia cell proliferation by BCR-ABL antisense oligodeoxynucleotides.** *Science* (1991) **253**:562-565.

14. SKORSKI T, NIEBOROWSKASKORSKA M, BARLETTA C, MALAGUARNERA L, SZCZYLIK C, CHEN ST, LANGE B, CALABRETTA B: **Highly efficient elimination of Philadelphia leukemic cells by exposure to bcr/abl antisense oligodeoxynucleotides combined with Mafosfamide.** *J. Clin. Invest.* (1993) **9**:194-202.

15. AKHTAR S, IVINSON AJ: **Therapies that make sense.** *Nature Genet.* (1993) **4**:215-217.

16. BAYEVER E, IVERSEN P, SMITH L, SPINOLO J, ZON G: **Guest editorial: systemic human antisense therapy begins.** *Antisense Res. Dev.* (1992) **2**:109-110.

17. SMITH LJ, MCCULLOCH EA, BENCHIMOL S: **Expression of the *p53* oncogene in acute myeloblastic leukemia.** *J. Exp. Med.* (1986) **164**:71-76.

18. MAHER LJ, DOLNICK BJ: **Specific hybridization arrest of dihydrofolate reductase mRNA *in vitro* using anti-sense RNA or anti-sense oligonucleotides.** *Arch. Biochem. Biophys.* (1987) **253**:214-220.

19. WALDER RY, WALDER JA: **Role of RNase H in hybrid-arrested translation by antisense oligonucleotides.** *Proc. Natl. Acad. Sci. USA* (1988) **85**:5011-5015.

20. LOKE SL, STEIN C, ZHANG XH, MORI K, NAKANISHI M, SUBASINGHE C, COHEN JS, NECKERS LM: **Characterization of oligonucleotide transport into living cells.** *Proc. Natl. Acad. Sci. USA* (1989) **86**:3474-3478.

21. YAKUBOV LA, DEEVA EA, ZARYTOVA VF, IVANOVA EM, RYTE AS, YURCHENKO LV, VLASSOV VV: **Mechanism of oligonucleotide uptake by cells: involvement of receptors?** *Proc. Natl. Acad. Sci. USA* (1989) **86**:6454-6458.

22. STEIN CA, TONKINSON JL, ZHANG LM, YAKUBOV L, GERVASONI J, TAUB R, ROSENBERG SA: **Dynamics of the internalization of phosphodiester oligodeoxynucleotides in HL60 cells.** *Biochemistry* (1993) **32**:4855-4861.

23. AKHTAR S, SHOJI Y, JULIANO RL: **Pharmaceutical aspects of the biological stability and membrane transport characteristics of antisense. In:** *Gene Regulation: Biology of Antisense RNA and DNA*. Erickson RP, Izant JG (Eds.). Raven Press Ltd., New York (1992) **1**:133-145.

24. SHAW JP, KENT K, BIRD J, FISHBACK J, FROEHLER B: **Modified deoxyoligonucleotides stable to exonuclease degradation in serum.** *Nucleic Acids Res.* (1991) **19**:747-750.

25. LETSINGER RL, ZHANG G, SUN DK, IKEUCHI T, SARIN PS: **Cholesteryl-conjugated oligonucleotides: synthesis, properties and activity as inhibitors of replication of human immunodeficiency virus in culture.** *Proc. Natl. Acad. Sci. USA* (1989) **86**:6553-6556.

26. CLARENCE JP, DEGOLS G, LEONETTI JP, MILHAUD P, LEBLEU B: **Delivery of antisense oligonucleotides by poly (L-lysine) conjugation and liposome encapsulation.** *Anti-Cancer Drug Design* (1993) **8**:81-94.

27. LEONETTI JP, MACHY P, DEGOLS G, LEBLEU B, LESERMAN L: **Antibody-targeted liposomes containing oligodeoxyonucleotides complementary to viral RNA selectively inhibit viral replication.** *Proc. Natl. Acad. Sci. USA* (1990) **87**:2448-2451.

28. MILLIGAN JF, JONES RJ, FROEHLER BC, MATTEUCCI MD: **Development of antisense therapeutics: Implications for cancer gene therapy.** *Annals New York Acad. Sci.* (1994) **716**:228-241.

29. EGUCHI Y, ITOH T, TOMIZAWA J: **Antisense RNA.** *Ann. Rev. Biochem.* (1991) **60**:631-652.
• A thorough review on antisense RNA.

30. KRYSTAL GW: **Regulation of eukaryotic gene expression by naturally occurring antisense RNA. In:** *Gene Regulation: Biology of Antisense RNA and DNA*. Erickson RP, Izant JG (Eds.). Raven Press Ltd., New York. (1992) **1**:11-20.

31. INOUYE M: **Antisense RNA: its function and applications in gene regulation - A review.** *Gene* (1988) **72**:25-34.
• A review of antisense RNA function and its applications in gene regulation.

32. WEINTRAUB HM: **Antisense RNA and DNA.** *Sci. Am.* (1990) **262**:40-48.

33. LEDWITH BJ, MANAM S, KRAYNAK AR, NICHOLS WW, BRADLEY MO: **Antisense-fos RNA causes partial reversion of the transformation phenotypes induced by the c-Ha-ras oncogene.** *Mol. Cell. Biol.* (1990) **10**:1545-1555.

34. SKLAR MD, THOMPSON E, WELSH MJ, LIEBERT M, HARNEY J, GROSSMAN HB, SMITH M, PROCHOWNIK EV: **Depletion of *c-myc* with specific antisense sequences reverses the transformed phenotype in *ras* oncogene-transformed NIH 3T3 cells.** *Mol. Cell. Biol.* (1991) **11**:3699-3710.

35. MUKHOPADHYAY T, TAINSKY M, CAVENDER AC, ROTH JA: **Specific inhibition of *K-ras* expression and tumorigenicity of lung cancer cells by antisense RNA.** *Cancer Res.* (1991) **51**:1744-1748.

36. ZHANG Y, MUKHOPADHYAY T, DONEHOWER LA, GEORGES RN, ROTH JA **Retroviral vector-mediated transduction of K-ras antisense RNA into human lung cancer cells inhibits expression of the malignant phenotype.** *Human Gene Ther.* (1993) **4**:451-460.

37. GEORGES RN, MUKHOPADHYAY T, ZHANG Y, YEN N, ROTH JAL: **Prevention of orthotopic human lung cancer growth by intratracheal instillation of a retroviral antisense K-ras construct.** *Cancer Res.* (1993) **53**:1743-1746.

38. KIBLER-HERZOG L, KELL B, ZON G, SHINOZUKA K, MIZAN S, WILSON WD: **Sequence-dependent effects in methylphosphonate deoxyribonucleotide double and triple helical complexes.** *Nucleic Acids Res.* (1990) **18**:3545-3555.

39. • MAHER LJ, WOLD B, DERVAN PB: **Inhibition of DNA binding proteins by oligonucleotide-directed triple helix formation.** *Science* (1989) **245**:725-730.

First report of inhibition of DNA binding proteins by oligonucleotide-directed triple helix formation.

40. HANVEY JC, SHIMIZU M, WELLS RD: **Site-specific inhibition of *EcoRI* restriction/modification enzymes by a DNA triple helix.** *Nucleic Acids Res.* (1990) **18**:157-161.

41. • COONEY M, CZERNUSZEWICZ G, POSTEL EH, FLINT SJ, HOGAN ME: **Site-specific oligonucleotide binding represses transcription of the human *c-myc* gene *in vitro*.** *Science* (1988) **241**:456-459.

First report of site-specific oligonucleotide binding represses transcription of the human *c-myc* gene *in vitro*.

42. ORSON FM, THOMAS DW, MCSHAN WM, KESSLER DJ, HOGAN ME: **Oligonucleotide inhibition of IL2Rα RNA transcription by promoter region collinear triplex formation in lymphocytes.** *Nucleic Acids Res.* (1991) **19**:3435-3441.

43. • GEE JE, MILLER DM: **Structure and applications of intermolecular DNA triplexes.** *Am. J. Med. Sci.* (1992) **304**:366-372.

A detailed review of structure and applications of intermolecular DNA triplexes.

44. ONO A, TS'O OP, KAN LS: **Triplex formation of oligonucleotides containing 2'-O-methylpseudo-isocytidine in substitution for 2'-deoxycytidine.** *J. Am. Chem. Soc.* (1991) **113**:4032-4033.

45. GAGNOR C, BERTRAND JR, THENET S, LEMAITRE M, MORVAN F, RAYNER B, MALVY C, LEBLEU B, IMBACH JL, PAOLETTI C: **Comparative study of α and β anomeric oligodeoxy-ribonucleotides in hybridization to mRNA and in cell-free translation inhibition.** *Nucleic Acids Res.* (1987) **15**:10507-10521.

46. HELENE C: **Control of gene expression by antisense and antigene oligonucleotide-intercalator conjugates. In:** *Gene Regulation: Biology of Antisense RNA and DNA.* Erickson RP, Izant JG (Eds.). Raven Press Ltd., New York (1992) **1**:109-118.

47. • KIM SH, CECH TR: **Three-dimensional model of the active site of the self-splicing rRNA precursor of Tetrahymena.** *Proc. Natl. Acad. Sci. USA* (1987) **84**:8788-8792.

First report on the three-dimensional model of the active site of ribozymes.

48. CECH TR: **Ribozymes and their medical implications.** *JAMA* (1988) **260**:3030-3034.

49. • VON AHSEN U, SCHROEDER R: **RNA as a catalyst: natural and designed ribozymes.** *BioEssays* (1993) **15**:299-307.

A thorough review on RNA as a catalyst: natural and designed ribozymes.

50. • KASHANI-SABET M, FUNATO T, TONE T, JIAO L, WANG W, YOSHIDA E, KASHFINN BI, SHITARA T, WU AM, MORENO JG, TRAWEEK ST, AHLERING TE, SCANLON KJ: **Reversal of the malignant phenotype by an anti-ras ribozyme.** *Antisense Res. Dev.* (1992) **2**:3-15.

A significant report on reversal of the malignant phenotype by an anti-oncogene ribozyme.

51. KOIZUMI M, KAMIYA H, OHTSUKA E: **Ribozymes designed to inhibit transformation of NIH3T3 cells by the activated *c-Ha-ras* gene.** *Gene* (1992) **117**:179-184.

52. SIOUD M, NATVIG JB, FORRE O: **Preformed ribozyme destroys tumour necrosis factor mRNA in human cells.** *J. Mol. Biol.* (1992) **223**:831-835.

53. WEERASINGHE M, LIEM SE, ASAD S, READ SE, JOSHI S: **Resistance to human immunodeficiency virus type 1 (HIV-1) infection in human CD4+ lymphocyte-derived cell lines conferred by using retroviral vectors expressing an HIV-1 RNA-specific ribozyme.** *J. Virol.* (1991) **65**:5531-5534.

54. LU D, CHATTERJEE S, BRAR D, WONG KK, Jr.: **Ribozyme-mediated *in vitro* cleavage of transcripts arising from the major transforming genes of human papillomavirus type 16.** *Cancer Gene Ther.* (1994) **1**:267-277.

55. •• HARRIS H: **How tumour suppressor genes were discovered.** *FASEB J.* (1993) **7**:978-979.

A thorough review on discovery of tumour suppressor genes.

56. ANDERSON MJ, STANBRIDGE EJ: **Tumour suppressor genes studied by cell hybridization and chromosome transfer.** *FASEB J.* (1993) **7**:826-833.

57. • DONEHOWER LA, HARVEY M, SLAGLE BL, MCARTHUR MJ, MONTGOMERY CA, BUTEL JS, BRADLEY A: **Mice deficient for *p53* are developmentally normal but susceptible to spontaneous tumors.** *Nature* (1992) **356**:215-221.

First report of the model of *p53* deficient transgenic mouse.

58. • WIMAN KG: **The retinoblastoma gene: role in cell-cycle control and cell differentiation.** *FASEB J.* (1993) **7**:841-845.

A detailed review about the role of *Rb* gene in cell-cycle control and cell differentiation.

59. •• HUANG HJS, YEE JK, SHEW JY, CHEN PL, BOOKSTEIN R, FRIEDMANN T, LEE EYHP, LEE WH: **Suppression of the neoplastic phenotype by replacement of the *Rb* gene in human cancer cells.** *Science* (1988) **242**:1563-1566.

A significant report of the tumor suppressor activity of the *Rb* gene.

60. •• BOOKSTEIN R, SHEW JY, CHEN PL, SCULLY P, LEE WH: **Suppression of tumorigenicity of human prostate carcinoma cells by replacing a mutated *Rb* gene.** *Science* (1990) **247**:712-715.

First report of suppression of tumourigenicity by restoring a wild type *Rb* gene.

61. • MONTENARH M: **Biochemical, immunological, and functional aspects of the growth-suppressor/oncoprotein p53.** *Crit. Rev. Oncog.* (1992) **3**:233-256.

A thorough review of p53 in biochemical, immunological, and functional aspects.

62. TOMINAGA O, HAMELIN R, REMVIKOS Y, SALMON RJ, THOMAS G: **p53 from basic research to clinical applications.** *Crit. Rev. Oncog.* (1992) **3**:257-282.

63. MERCER WE: **Cell-cycle regulation and the p53 tumour suppressor protein.** *Crit. Rev. Eukaryot. Gene Expr.* (1992) **2**:251-263.

64. KUERBITZ SJ, PLUNKETT BS, WALSH WV, KASTAN MB: **Wild-type p53 is a cell-cycle checkpoint determinant following irradiation.** *Proc. Natl. Acad. Sci. USA* (1992) **89**:7491-7495.

65. • FIELDS S, JANG SJ: **Presence of a potent transcription-activating sequence in the p53 protein.** *Science* (1990) **249**:1046-1049.
First report of presence of a potent transcription-activating sequence in the p53 protein.

66. MIETZ JA, UNGER T, HUIBREGTSE JM, HOWLEY PM: **The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein.** *EMBO J.* (1992) **11**:5013-5020.

67. • WILCOCK D, LANE DP: **Localization of p53, retinoblastoma, and host replication proteins at sites of viral replication in herpes-infected cells.** *Nature* (1991) **349**:429-431.
A significant report of involvement of the p53 and RB proteins in DNA replication.

68. BARGONETTI J, FRIEDMANN PN, KERN SE, VOGELSTEIN B, PRIVES C: **Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication.** *Cell* (1991) **65**:1083-1091.

69. • YONISH-ROUACH E, RESNITZKY D, LOTEM J, SACHS L, KIMCHI A, OREN M: **Wild-type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6.** *Nature* (1991) **352**:345-347.
First report of the p53 protein as an inducer of apoptosis.

70. SHAW P, BOVEY R, TARDY S, SAHLI R, SORDAT B, COSTA J: **Induction of apoptosis by wild-type p53 in a human colon tumour-derived cell line.** *Proc. Natl. Acad. Sci. USA* (1992) **89**:4495-4499.

71. RYAN JJ, DANIH R, GOTTLIEB CA, CLARKE M: **Cell-cycle analysis of p53-induced cell death in murine erythroleukemia cells.** *Mol. Cell. Biol.* (1993) **13**:711-719.

72. •• FINLAY CA, HINDS PW, LEVINE AJ: **The *p53* proto-oncogene can act as a suppressor of transformation.** *Cell* (1989) **57**:1083-1093.
A significant report of the p53 protein as a tumour suppressor.

73. • BAKER SJ, MARKOWITZ S, FEARSON ER, VILLSON JKV, VOGELSTEIN B: **Suppression of human colorectal carcinoma cell growth by wild-type *p53*.** *Science* (1990) **249**:912-915.
First report of tumour suppressor activity of the wild-type *p53* gene.

74. CHENG J, YEE JK, YEARGIN J, FRIEDMANN T, HAAS M: **Suppression of acute lymphoblastic leukemia by the human wild-type *p53* gene.** *Cancer Res.* (1992) **52**:222-226.

75. TAKAHASHI T, CARBONE D, TAKAHASHI T, NAU MM, HIDA T, LINNOILA I, UEDA R, MINNA JD: **Wild-type but not mutant p53 suppresses the growth of human lung cancer cells bearing multiple genetic lesions.** *Cancer Res.* (1992) **52**:2340-2343.

76. QUINLAN DC, DAVIDSON AG, SUMMERS CL, WARDEN HE, DOSHI HM: **Accumulation of p53 protein correlates with a poor prognosis in human lung cancer.** *Cancer Res.* (1992) **52**:4828-4831.

77. HORIO Y, TAKAHASHI T, KUROISHI T, HIBI K, SUYAMA M, NIIMI T: **Prognostic significance of *p53* mutations and 3P deletions in primary resected non-small cell lung cancer.** *Cancer Res.* (1993) **53**:1-4.

78. SHRIVASTOVA S, ZOU A, PIROLLO K, BLATTNER S, CHANG E: **Germline transmission of a mutated *p53* gene in a cancer prone family with Li-Fraumeni syndrome.** *Nature* (1990) **348**:747-749.

79. • HARPER JW, ADAM GR, WEI N, KEYOMARSI K, ELLEDGE SJ: **The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinase.** *Cell* (1993) **75**:805-816.
A significant report of identification of the p21 protein as an inhibitor of G1 cyclin-dependent kinase.

80. • EL-DEIRY WS, TOKINO T, VELCULESCU VE, LEVY DB, PARSONS R, TRENT JM, LIN D, MERCER WE, KINZIER KW, VOGELSTEIN B: ***WAF1*, a potential mediator of p53 tumor suppression.** *Cell* (1993) **75**:817-825.
First report on *WAF1*, a potential mediator of p53 tumour suppression.

81. ROEMER K, FRIEDMANN T: **Mechanisms of action of the p53 tumour suppressor and prospects for cancer gene therapy by reconstitution of p53 function.** *Annals New York Acad. Sci.* (1994) **716**:265-280.

82. GOYETTE MC, CHO K, FASCHING CL, LEVY DB, KINZLER KW, PARASKEVA C, VOGELSTEIN B, STANBRIDGE EJ: **Progression of colorectal cancer is associated with multiple tumour suppressor gene defects but inhibition of tumorigenicity is accomplished by correction of any single defect *via* chromosome transfer.** *Mol. Cell. Biol.* (1992) **12**:1387-1395.

83. • LEONE A, FLATOW U, KING CR, SANDEEN MA, MARGULIES MK, LIOTTA LA, STEEG PS: **Reduced tumour incidence, metastatic potential, and cytokine responsiveness of nm23-transfected melanoma cells.** *Cell* (1991) **65**:25-35.
A significant report of the tumour suppression activity of *nm23*.

84. FLORENES VA, AAMDAL S, MYKLEBOST O, MAELANDSMO GM, BRULAND OS, FODSTAD O: **Levels of nm23 messenger RNA in metastatic melanomas inverse correlation to disease prognosis.** *Cancer Res.* (1992) **52**:6088-6091.

85. •• KAMB A, GRUIS NA, WEAVER-FELDHAUS J, LIU Q, HARSHMAN K, TAVTIGIAN SV, STOCKERT E, DAY RS III, JOHNSON BE, SKOLNICK MH: **A cell cycle regulator potentially involved in genesis of many tumour types.** *Science* (1994) **264**:436-440.
First report of *p16* and *p15* as major tumour suppressors (MTS).

86. NOBORI T, MIURA K, WU DJ, LOIS A, TAKABAYASHI K, CARSON DA: **Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers.** *Nature* (1994) **368**:753-756.

87. •• MIKI Y, SWENSEN J, SHATTUCK-EIDENS D, FUTREAL PA, HARSHMAN K, TAVTIGIAN S, LIU Q, COCHRAN C, BENNETT LM, DING W, BELL R, ROSENTHAL J, HUSSEY C, TRAN T, MCCLURE M, FRYE C, HATTIER T, PHELPS R, HAUGEN-STRANO A, KATCHER H, YAKUMO K, GHOLAMI Z, SHAFFER D, STONE S, BAYER S, WRAY C, BOGDEN R, DAYANANTH P, WARD J, TONIN P, NAROD S, BRISTOW PK, NORRIS FH, HELVERING L, MORRISON P, ROSTECK P, LAI M, BARRETT JC, LEWIS C, NEUHAUSEN S, CANNON-ALBRIGHT L, GOLDGAR D, WISEMAN R, KAMB A, SKOLNICK MH: **A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*.** *Science* (1994) **266**:66-71.
First report of identification of the *BRCA1* gene.

88. HANNON G, BEACH D: **p15INK4B is a potential effector of TGF-β-induced cell cycle arrest.** *Nature* (1994) **371**:257-261.

89. MARX J: **A challenge to *p16* gene as a major tumour suppressor.** *Science* (1994) **264**:1846.

90 ZHANG S-Y, KLEIN-SZANTO AJP, SAUTER ER, SHAFARENKO M, MITSUNAGA S, NOBORI T, CARSON DA, RIDGE JA, GOODROW TL: **Higher frequency of alternations in the *p16/CDKN2* gene in squamous cell carcinoma cell lines than in primary tumours of the head and neck.** *Cancer Res.* (1994) **54**:5050-5053.

91 JEN J, HARPER W, BIGNER SH, BIGNER DD, PAPADOPOULOS N, MARKOWITZ S, WILLSON JK V, KINZLER KW, VOGELSTEIN B: **Deletion of p16 and p15 genes in brain tumors.** *Cancer Res.* (1994) **54**:6353-6358.

92. WASHIMI O, NAGATAKE M, OSADA H, UEDA R, KOSHIKAWA T, SEKI T, TAKAHASHI T, TAKAHASHI T: ***In vivo* occurrence of *p16 (MTS1)* and *p15 (MTS2)* alterations preferentially in non-small cell lung cancers.** *Cancer Res.* (1995) **55**:514-517.

93. HUSSUSSIAN CJ, STRUEWING JP, GOLDSTEIN AM, HIGGINS PAT, ALLY DS, SHEAHAN MD, CLARK WH JR, TUCKER MA, DRACOPOLI NC: **Germline *p16* mutation in familial melanoma.** *Nature Genet.* (1994) **8**:15-21.

94. CAI D W, MUKHOPADHYAY T, LIU Y, FUJIWARA T, ROTH JA: **Stable expression of the wild-type p53 gene in human lung cancer cells after retrovirus-mediated gene transfer.** *Human Gene Ther.* (1993) **4**:617-624.

95. FUJIWARA T, GRIMM EA, MUKHOPADHYAY T, CAI DW, OWEN-SCHAUB LB, ROTH JA: **A retroviral wild-type p53 expression vector penetrates human lung cancer spheroids and inhibits growth by inducing apoptosis.** *Cancer Res.* (1993) **53**:4129-4133.

96. CASSON AG, MUKHOPADHYAY T, CLEARY KR, RO JY, LEVINE B, ROTH JA: **p53 mutations in Barrett's epithelium and esophageal cancer.** *Cancer Res.* (1991) **51**:4495-4499.

97. CHUNG KY, MUKHOPADHYAY T, KIM J, CASSON A, RO JY, GOEPFERT H, HONG WK, ROTH JA: **Discordant p53 gene mutations in primary head and neck cancers and corresponding second primary cancers of the aerodigestive tract.** *Cancer Res.* (1993) **53**:1676-1683.

98. • ZHANG W-W, FANG X, BRANCH CD, MAZUR W, FRENCH BA, ROTH JA: **Generation and identification of recombinant adenovirus by liposome-mediated transfection and PCR analysis.** *BioTechniques* (1993) **15**:868-872.

First report of generation of recombinant *p53* adenovirus.

99. • ZHANG W-W, FANG X, MAZUR W, FRENCH BA, GEORGES RN, ROTH JA: **High-efficiency gene transfer and high-level expression of wild-type p53 in human lung cancer cells mediated by recombinant adenovirus.** *Cancer Gene Ther.* (1994) **1**:5-13.

A significant report of the recombinant *p53* adenovirus and its application in lung cancer suppression.

100. WANG J, BUCANA CD, ROTH JA, ZHANG W-W: **Apoptosis induced in human osteosarcoma cells is one of the mechanisms for the cytocidal effect of Ad5CMV-*p53*.** *Cancer Gene Ther.* (1995) **1**:9-17.

101. WILLS KN, MANEVAL DC, MENZEL P, HARRIS MP, SUTJIPTO S, VAILLANCOURT M-T, HUANG W-M, JOHNSON DE, ANDERSON SC, WEN SF, BOOKSTEIN R, SHEPARD HM, GREGORY RJ: **Development and characterization of recombinant adenoviruses encoding human p53 for gene therapy of cancer.** *Human Gene Ther.* (1994) **5**:1079-1088.

102. LIU TJ, ZHANG W-W, TAYLOR DL, ROTH JA, GOEPFERT H, CLAYMAN GL: **Growth suppression of human head and neck cancer cells by the introduction of a wild-type p53 gene *via* a recombinant adenovirus.** *Cancer Res.* (1994) **54**:3662-3667.

103. MOOLTEN FL: **Drug sensitivity ('suicide') genes for selective cancer chemotherapy.** *Cancer Gene Ther.* (1994) **1**:279-287.

104. MAXWELL IH, MAXWELL F, GLODE LM: **Regulated expression of a diphtheria toxin A-chain gene transfected into human cells: possible strategy for inducing cancer cell suicide.** *Cancer Res.* (1986) **46**:4660-4664.

105. • BREITMAN M L, CLAPOFF S, ROSSANT J, TSUI L C, GLODE LM, MAXWELL IH, BERNSTEIN A: **Genetic ablation: programmed lineage suicide by tissue-specific expression of the diphtheria toxin A gene in transgenic mice.** *Science* (1987) **238**:1563-1565.

First report of tissue-specific expression of a toxin gene *in vivo*.

106. PALMITER RD, BEHRINGER RR, QUAIFE CJ, MAXWELL F, MAXWELL IH, BRINSTER RL: **Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene.** *Cell* (1987) **50**:435-443.

107. YAMAIZUMI M, MEKADA E, UCHIDA T, OKADA Y: **One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell.** *Cell* (1978) **15**:245-250.

108. MAXWELL IH, GLODE LM, MAXWELL F: **Expression of diphtheria toxin A-chain in mature B-cells: a potential approach to therapy of B-lymphoid malignancy.** *Leuk. Lymphoma* (1992) **7**:457-462.

109. PURI RK, OGATA M, LELAND P, FELDMAN GM, FITZGERALD D, PASTAN I: **Expression of high-affinity interleukin 4 receptors on murine sarcoma cells and receptor-mediated cytotoxicity of tumour cells to chimeric protein between interleukin 4 and *Pseudomonas* exotoxin.** *Cancer Res.* (1991) **51**:3011-3017.

110. RAO L, DEBBAS M, SABBATINI P, HOCKENBERY D, KORSMEYER S, WHITE E: **The adenovirus E1A proteins induce apoptosis which is inhibited by the E1B 19K and Bcl-2 proteins.** *Proc. Natl. Acad. Sci. USA* (1992) **89**:7742-7746.

111. DEBBAS M, WHITE E: **Wild-type p53 mediates apoptosis by E1A which is inhibited by E1B.** *Gene Dev.* (1993) **7**:546-554.

112. YU D, WOLF JK, SCANLON M, PRICE JE, HUNG MC: **Enhanced c-*erb*B-2/*neu* expression in human ovarian cancer cells correlates with more severe malignancy that can be suppressed by E1A.** *Canc. Res.* (1993) **53**:891-898.

113. ZHANG Y, YU D, XIA W, HUNG MC: ***HER-2/neu*-targeting cancer therapy *via* adenovirus-mediated E1A delivery in an animal model.** *Oncogene* (1995). In press.

114. ELION GB, FURMAN PA, FYFE JA, DE MIRANDA P, BEAUCHAMP L, SCHAEFFER HJ: **Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine.** *Proc. Natl. Acad. Sci. USA* (1977) **74**:5716-5720.

115. • DAVIDSON RL, KAUFMAN ER, CRUMPACKER CS, SCHNIPPER LE: **Inhibition of herpes simplex virus transformed and nontransformed cells by acycloguanosine: mechanisms of uptake and toxicity.** *Virology* (1981) **113**:9-19.

A mechanism study of acycloguanosine as anti-HSV agent.

116. MOOLTEN FL, WELLS JM: **Curability of tumours bearing herpes thymidine kinase genes transferred by retroviral vectors.** *J. Natl. Cancer Inst.* (1990) **82**:297-300.

117. •• CULVER KW, RAM Z, WALLBRIDGE S, ISHII H, OLDFIELD EH, BLAESE RM: **Vector-producer cells for treatment of**

experimental brain tumours. *Science* (1992) **256**:1550-1552.

A significant report of treatment of brain tumour model with HSV-*tk* gene transfer with systemic administration of GCV.

118 BI WL, PARYSEK LM, WARNICK R, STAMBROOK PJ: ***In vitro* evidence that metabolic cooperation is responsible for the bystander effect observed with HSV-*tk* retroviral gene therapy.** *Human Gene Ther.* (1993) **4**:725-731.

119. OLDFIELD EH: **Gene therapy for the treatment of brain tumours using intratumoral transduction with the thymidine kinase gene and intravenous ganciclovir.** *Human Gene Ther.* (1993) **4**:36-69.

120. • HUBER BE, RICHARDS CA, KRENITSKY TA: **Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: An innovative approach for cancer therapy.** *Proc. Natl. Acad. Sci. USA* (1991) **88**:8039-8043.

A significant report of design of tissue-specific expression of a prodrug-activation gene for gene therapy of cancer.

121. MULLEN CA, KILSTRUP M, BLAESE RM: **Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system.** *Proc. Natl. Acad. Sci. USA* (1992) **89**:33-37.

122. GARVER RI, Jr., GOLDSMITH KT, RODU B, HU PC, SORSCHER EJ, CURIEL DT: **Strategy for achieving selective killing of carcinomas.** *Gene Ther.* (1994) **1**:46-50.

123. MANOME Y, ABE M, HAGEN MF, FINE HA, KUFE DW: **Enhancer sequences of the DF3 gene regulate expression of the herpes simplex virus thymidine kinase gene and confer sensitivity of human breast cancer cells to ganciclovir.** *Cancer Res.* (1994) **54**:5408-5413.

124. SMITH MJ, ROUSCULP MD, GOLDSMITH KT, CURIEL DT, GARVER RI, Jr.: **Surfactant protein A-directed toxin gene kills lung cancer cells *in vivo*.** *Human Gene Ther.* (1994) **5**:29-35.

125. DIMAIO JM, CLARY BM, VIA DF, COVENEY E, PAPPAS TN, LYERLY HK: **Directed enzyme pro-drug gene therapy for pancreatic cancer *in vivo*.** *Surgery* (1994) **116**:205-213.

126. WEI M-X, TAMIYA T, CHASE M, BOVIATSIS EJ, CHANG TKH, KOWALL NW, HOCHBERG FH, WAXMAN DJ, BREAKEFIELD XO, CHIOCCA EA: **Experimental tumor therapy in mice using the cyclophosphamide-activating cytochrome P450 2B1 gene.** *Human Gene Ther.* (1994) **5**:969-978.

127. WEICHSELBAUM RR, HALLAHAN DE, BECKETT MA, MAUCERI HJ, LEE H, SUKHATME VP, KUFE DW: **Gene therapy targeted by by radiation preferentially radiosensitizes tumour cells.** *Cancer Res.* (1994) **54**:4266-4269.

128. • GOTTESMAN MM, GERMANN UA, AKSENTIJEVICH I, SUGIMOTO Y, CARDARELLI CO, PASTAN I: **Gene therapy of drug resistance genes: implications for cancer therapy.** *Annals New York Acad. Sci.* (1994) **716**:126-138.

A recent review of *MDR* genes and their application in cancer gene therapy.

129. PASTAN I, GOTTESMAN MM: **Multiple-drug resistance in human cancer.** *New Engl. J. Med.* (1987) **316**:1388-1393.

130. SHEN DW, CARDARELLI C, HWANG J, CORNWELL MM, RICHERT N, ISHII SI, PASTAN I, GOTTESMAN MM: **Multiple drug-resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin, or vinblastine show changes in expression of specific proteins.** *J. Biol. Chem.* (1986) **261**:7762-7770.

131. • RONINSON IB, CHIN JE, CHOI K, GROS P, HOUSMAN DE, FOJO A, SHEN DW, GOTTESMAN MM, PASTAN I: **Isolation of the human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells.** *Proc. Natl. Acad. Sci. USA* (1986) **83**:4538-4542.

A significant report of isolation of the human *MDR1* gene.

132. CHEN CJ, CHIN JE, UEDA K, CLARK D, PASTAN I, GOTTESMAN MM, RONINSON IB: **Internal duplication and homology with bacterial transport protein in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells.** *Cell* (1986) **47**:381-389.

133. • JURANA PF, ZASTAWNY RL, LING V: **P-glycoprotein: Multidrug-resistance and a superfamily of membrane-associated transport proteins.** *FASEB J.* (1989) **3**:2583-2592.

A detailed review on multidrug-resistance and a superfamily of membrane-associated transport proteins.

134. GOTTESMAN MM, PASTAN I: **Biochemistry of multidrug resistance mediated by the multidrug transporter.** *Ann. Rev. Biochem.* (1993) **62**:385-427.

135. RIORDAN JR, DEUCHARS K, KARTNER N, ALON N, TRENT J, LING V: **Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines.** *Nature* (1985) **316**:817-819.

136. GROS P, NERIAH YB, CROOP JM, HOUSMAN DE: **Isolation and expression of a complementary DNA that confers multidrug resistance.** *Nature* (1986) **323**:728-731.

137. SHEN DW, FOJO A, RONINSON IB, CHIN JE, SOFFIR P, PASTAN I, GOTTESMAN MM: **Multidrug resistance of DNA-mediated transformants is linked to transfer of the human mdr1 gene.** *Mol. Cell. Biol.* (1986) **6**:4039-4044.

138. • GALSKI H, SULLIVAN M, WILLINGHAM MC, CHIN KV, GOTTESMAN MM, PASTAN I, MERLINO GT: **Expression of a human multidrug resistance cDNA (MDR1) in the bone marrow of transgenic mice: Resistance to daunomycin-induced leukopenia.** *Mol. Cell. Biol.* (1989) **9**:4357-4363.

A study of the *MDR1* transgenic mouse model.

139. MORROW CS, COWAN KH: **Mechanisms and clinical significance of multidrug resistance.** *Oncology* (1988) **2**:55-67.

140. BECK WT: **Mechanisms of multidrug resistance in human tumour cells. The roles of P-glycoprotein, DNA topoisomerase II, and other factors.** *Cancer Treat. Rev.* (1990) **17**(Suppl. A):11-20.

141. FINE RL, PATEL J, CHABNER BA: **Phorbol esters induce multidrug resistance in human breast cancer cells.** *Proc. Natl. Acad. Sci. USA* (1988) **85**:582-586.

142. TAYLOR CW, BRATTAIN MG, YEOMAN LC: **Occurrence of cytosolic protein and phosphoprotein changes in human colon tumor cells with the development of resistance to mitomycin C.** *Cancer Res.* (1985) **45**:4422-4427.

143. WANG AL, TEW KD: **Increased glutathione-*S*-transferase activity in a cell line with acquired resistance to nitrogen mustards.** *Cancer Treat. Rep.* (1985) **69**:677-682.

144. MCGOWN AT, FOX BW: **A proposed mechanism of resistance to cyclophosphamide and phosphoramide mustard in a Yoshida cell line *in vitro*.** *Cancer Chemother. Pharmacol.* (1986) **17**:223-227.

145. ROBSON CN, LEWIS AD, WOLF CR, HAYES JD, HALL A, PROCTOR SJ, HARRIS AL, HICKSON ID: **Reduced levels of drug-induced DNA cross-linking in nitrogen mustard-resistant Chinese hamster ovary cells expressing elevated glutathione-*S*-transferase activity.** *Cancer Res.* (1987) **47**:6022-6027.

146. MORROW CS, COWAN KH: **Multidrug resistance associated with altered topoisomerase II activity-topoisomerase II as targets for rational drug design.** *J. Natl. Cancer Inst.* (1990) **82**:638-639.

147. DEFFIE AM, BATRA JK, GOLDENGERG JG: **Direct correlation between DNA topoisomerase II activity and cytotoxicity in adriamycin-sensitive and -resistant P388 leukemia cell lines.** *Cancer Res.* (1989) **49**:58-62.

148. SAVAS B, COLE SPC, AKOGLU TF, PROSS HF: **P-glycoprotein-mediated multidrug resistance and cytotoxic effector cells.** *Nat. Immun.* (1992) **11**:177-192.

149. MICKISCH GH, AKSENTIJEVICH I, SCHOENLEIN PV, GOLDSTEIN LJ, GALSKI H, STAHLE C, SACHS DH, PASTAN I, GOTTESMAN MM: **Transplantation of bone marrow cells from transgenic mice expressing the human MDR1 gene results in long-term protection against the myelosuppressive effect of chemotherapy in mice.** *Blood* (1992) **79**:1-7.

150. PODDA S, WARD M, HIMELSTEIN A, RICHARDSON C, FLOR-WEISS E, SMITH L, GOTTESMAN MM, PASTAN I, BANK A: **Transfer and expression of the human multiple drug resistance (MDR) gene into live mice.** *Proc. Natl. Acad. Sci. USA* (1992) **89**:9676-9680.

151. • SORRENTINO BP, BRANDT SJ, BODINE D, GOTTESMAN MM, PASTAN I, CLINE A, NIENHUIS AW: **Retroviral transfer of the human MDR1 gene permits selection of drug-resistant bone marrow cells *in vivo*.** *Science* (1992) **257**:99-103.

A significant report of *in vivo* selection of drug-resistant bone marrow cells after transfer with the *MDR1* gene: implication for chemoprevention.

152. HANANIA EG, FU SQ, RONINSON I, ZU Z, GOTTESMAN MM, HEGEWISCH-BECKER S, ANDREEFF M, DEISSEROTH AB: **Resistance to Taxol chemotherapy engineered by safety-modified retroviruses containing an MDR-1 transcription unit.** *Gene Ther.* (1995). In press.

153. HANANIA EG, DEISSEROTH AB: **Serial transplantation shows that early hematopoietic precursor cells are transduced by MDR-1 retroviral vector in a mouse gene therapy model.** *Cancer Gene Ther.* (1994) **1**:21-25.

154. COREY CA, DESILVA AD, HOLLAND CA, WILLIAMS DA: **Serial transplantation of methotrexate-resistant bone marrow: protection of murine recipients from drug toxicity by progeny of transduced stem cells.** *Blood* (1990) **75**:337-343.

155. SURESH A, TUNG E, MOREB J, JAMES RZ: **Role of manganese superoxide dismutase in radioprotection using gene transfer studies.** *Cancer Gene Ther.* (1994) **1**:85-90.

156. •• ROSENBERG SA, AEBERSOLD P, CORNETTA K, KASID A, MORGAN RA, MOEN R, KARSON EM, LOTZE MT, YANG JC, TOPALIAN SL, MERINO MJ, CULVER K, MILLER AD, BLAESE RM, ANDERSON WF: **Gene transfer into humans: immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction.** *New Engl. J. Med.* (1990) **323**:570-578.

First report of *ex vivo* gene transfer into human.

157. BLAESE RM, ANDERSON WF: **The ADA human gene therapy clinical protocol.** *Human Gene Ther.* (1990) **1**:327-329.

158. • ROSENBERG SA, SPIESS P, LAFRENIERE R: **A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes.** *Science* (1986) **233**:1318-1321.

An introduction about application of TIL for cancer therapy.

159. MUUL LM, SPIESS PJ, DIRECTOR, EP, ROSENBERG SA: **Identification of specific cytolytic immune responses against autologous tumor in humans bearing malignant melanoma.** *J. Immunol.* (1987) **138**:989-995.

160. BARTH RJ, BOCK SN, MULE JJ, ROSENBERG SA: **Unique murine tumor associated antigens identified by tumor-infiltrating lymphocytes.** *J. Immunol.* (1990). **144**:1531-1537.

161. BARTH RJ, MULE JJ, SPIESS PJ, ROSENBERG SA: **Interferon gamma and tumor necrosis factor have a role in tumor regressions mediated by murine CD8$^+$ tumor-infiltrating lymphocytes.** *J. Exp. Med.* (1991). **173**:647-658.

162. TOPALIAN SL, SOLOMON D, ROSENBERG SA: **Tumor-specific cytolysis by lymphocytes infiltrating human melanoma.** *J. Immunol.* (1989) **142**:3714-3725.

163. FISHER B, PAKARD BS, READ EJ, CARRASQUILLO JA, CARTER CS, TOPALIAN S, YANG JC, YOLLES P, LARSON SM, ROSENBERG SA: **Tumor localization of adoptively transferred Indium-111 labeled tumor infiltrating lymphocytes in patients with metastatic melanoma.** *J. Clin. Oncol.* (1989) **7**:250-261.

164. GRIFFITH KD, READ EJ, CARRASQUILLO JA, CARTER CS, YANG JC, FISHER B, AEBERSOLD P, PACKARD BS, YU MY, ROSENBERG SA: ***In vivo* distribution of adoptively transferred Indium-111 labeled tumor-infiltrating lymphocytes and peripheral blood lymphocytes in patients with metastatic melanoma.** *J. Natl. Cancer Inst.* (1989) **81**:1709-1717.

165. ROSENBERG SA, PACKARD BS, AEBERSOLD PM, SOLOMON D, TOPALIAN SL, TOY ST, SIMON P, LOTZE MT, YANG JC, SEIPP CA, SIMPSON C, CARTER C, BOOK S, SCHWARTZENTRUBER D, WEI JP, WHITE DE: **Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma: a preliminary report.** *New Engl. J. Med.* (1988) **319**:1676-1680.

166. KASID A, MORECKI S, ABERSOLD P, CORNETTA K, CULVER K, FREEMAN S, DIRECTOR E, LOTZE MT, BLAESE RM, ANDERSON WF, ROSENBERG SA: **Human gene transfer: characterization of human tumor-infiltrating lymphocytes as vehicles for retroviral-mediated gene transfer in man.** *Proc. Natl. Acad. Sci. USA* (1990) **87**:473-477.

167. RILL DR, MOEN RC, BUSCHLE M, BARTHOLOMEW C, FOREMAN NK, MIRRO J, KRANCE RA, IHLE JN, BRENNER MK: **An approach for the analysis of relapse and marrow reconstitution after autologous marrow transplantation using retrovirus-mediated gene transfer.** *Blood* (1992) **79**:2694-2700.

168. DIESSEROTH AB: **Use of two retroviral markers to test relative contribution of marrow and peripheral blood autologous cells to recovery after preparative therapy.** *Human Gene Ther.* (1993) **4**:71-85.

169. ASHER AL, MULE JJ, REICHERT CM, SHILONI E, ROSENBERG SA: **Studies on the anti-tumor efficacy of systemically administered recombinant tumor necrosis factor**

against several murine tumors *in vivo*. *J. Immunol.* (1987) **138**:963-974.

170. ROSENBERG SA: **Immunization of cancer patients using autologous cancer cells modified by insertion of the gene for tumor necrosis factor.** *Human Gene Ther.* (1992) **3**:57-73.

171. ROSENBERG SA: **Gene therapy for cancer. In:** *Important Advances in Oncology*. DeVita VT, Jr., Hellman S, Rosenberg SA (Eds.). JB Lippincott Company, Philadelphia (1992):17-38.

172. HWU P, YANNELLI J, KRIEGLER M, ANDERSON WF, PEREZ C, CHIANG Y, SCHEARZ S, COWHERD R, DELGADO C, MULE J, ROSENBERG SA: **Functional and molecular characterization of tumor-infiltrating lymphocytes transduced with tumor necrosis factor-α cDNA for the gene therapy of cancer in humans.** *J. Immunol.* (1993) **150**:4104-4115.

173. ESHHAR Z: **Redirection of effector lymphocytes to tumor cells using chimeric receptors with antibody specificity.** *Cancer Gene Ther.* (1994) **1**:139.

174. ESHHAR Z, WAKS T, GROSS G, SCHINDLER D: **Specific activation and targeting of cytotoxic lymphocytes through chimeric single-chains consisting of antibody-binding domains and the γ or ζ subunits of the immunoglobulin and T-cell receptors.** *Proc. Natl. Acad. Sci. USA* (1993) **90**:720-724.

175. MORITZ D, WELS W, MATTERN J, GRONER B: **Cytotoxic T-lymphocytes with a grafted recognition specificity for ERBB2-expressing tumor cells.** *Proc. Natl. Acad. Sci. USA* (1994) **91**:4318-4322.

176. OETTGEN HF, OLD LJ: **The history of cancer immunotherapy. In:** *Biologic Therapy of Cancer*. DeVita VT, Hellman S, Rosenberg SA (Eds.). JB Lippincott, Philadelphia (1991):87-119.

177. VAAGE J: **Peritumor interleukin-2 causes systemic therapeutic effect *via* interferon-γ induction.** *Int. J. Cancer* (1991) **49**:598-600.

178. HULAND E, HULAND H, HEINZER H: **Interleukin-2 by inhalation: local therapy for metastatic renal cell carcinoma.** *J. Urol.* (1992) **147**:344-348.

179. COLOMBO M, MODESTI A, PARMIANI G, FORNI G: **Local cytokine availability elicits tumor rejection and systemic immunity through granulocyte-T-lymphocyte cross-talk.** *Cancer Res.* (1992) **52**:4853-4857.

180. • TEPPER RI, MULE JJ: **Experimental and clinical studies of cytokine gene-modified tumor cells.** *Human Gene Ther.* (1994) **5**:153-164.
A detailed review on cytokine gene-modified tumor cells as tumor vaccines.

181. LOTZE MT: **Role of IL-4 in the antitumor response. In:** *IL-4: Structure and Function*. Spits H (Ed.). CRC Press, Boca Raton, FL (1991) **147**:2950-2956.

182. TEPPER RI, PATTENGALE PK, LEDER P: **Murine interleukin-4 displays potent antitumor activity *in vivo*.** *Cell* (1989) **57**:503-512.

183. • GOLUMBEK PT, LAZENBY AJ, LEVITSKY HI, JAFFEE LM, KARASUYAMA H, BAKER M, PARDOLL DM: **Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4.** *Science* (1991) **254**:713-716.
An early study of the cytokine gene-modified tumour vaccine.

184. PIPPIN BA, ROSENSTEIN M, JACOB WF, CHIANG Y, LOTZE MT: **Local IL-4 delivery enhances immune reactivity to murine tumors: Gene therapy in combinantion with IL-2.** *Cancer Gene Ther.* (1994) **1**:35-42.

185. • SMITH KA: **Interleukin-2: inception, impact, and implications.** *Science* (1988) **240**:1169-1176.
A detailed review of IL-2.

186. GRIMM EA, MAZUMDER A, ZHANG HZ, ROSENBERG SA: **Lymphokine-activated killer phenomenon. Lysis of natural killer resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes.** *J. Exp. Med.* (1982) **155**:1823-1841.

187. FEARON E, PARDOLL D, ITAYA T, GOLUMBEK P, LEVITSKY H, SIMONS J, KARASUYAMA H, VOGELSTEIN B, FROST P: **Interleukin-2 production by tumor cells bypasses T-helper function in the generation of an antitumor response.** *Cell* (1990) **60**:397-403.

188. GANSBACHER B, ZIER K, DANIELS B, CRONIN K, BANNERJI R, GILBOA E: **Interleukin-2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity.** *J. Exp. Med.* (1990) **172**:1217-1224.

189. BUBENIK J, SIMOVA J, JANDLOVA T: **Immunotherapy of cancer using local administration of lymphoid cells expressing IL-2 cDNA and constitutively producing IL-2.** *Immunol. Lett.* (1990) **23**:287-292.

190. PORGADOR A, GANSBACHER B, BANNERI R, TZEHOVAL E, GILBOA E, FELDMAN M, EISENBACH L: **Anti-metastatic vaccination of tumor-bearing mice with IL-2-gene-inserted tumor cells.** *Int. J. Cancer* (1993) **53**:471-477.

191. TSAI SCJ, GANSBACHER B, TAIT L, MILLER FR, HEPPNER GH: **Induction of antitumor immunity by interleukin-2 gene-transduced mouse mammary tumor cells *versus* transduced mammary stromal fibroblasts.** *J. Natl. Cancer Inst.* (1993) **85**:546-553.

192. GANSBACHER B, ZIER K, CRONIN K, HANTZOPOULOS PA, BOUCHARD B, HOUGHTON A, GILBOA, E, GOLDE D: **Retroviral gene transfer induced constitutive expression of interleukin-2 or interferon-γ in irradiated human melanoma cells.** *Blood* (1992) **80**:2817-2825.

193. HADDADA H, RAGOT T, CORDIER L, DUFFOUR MT, PERRICADUET M: **Adenoviral interleukin-2 gene transfer into p815 tumor cells abrogates tumorigenicity and induces antitumoral immunity in mice.** *Human Gene Ther.* (1993) **4**:703-711.

194. GASTL G, FINSTAD CL, GUARINI A, BOSL G, GILBOA E, BANDER NH, GANSBACHER B: **Retroviral vector-mediated lymphokine gene transfer into human renal cancer cells.** *Cancer Res.* (1992) **52**:6229-6236.

195. ZIER KS, SALVADORI S, CRONIN KC, GANSBACHER B: **Vaccination with IL-2-secreting tumor cells stimulates the generation of IL-2-responsive T-cells and prevents the development of unresponsiveness.** *Cancer Gene Ther.* (1994) **1**:43-50.

196. • FOA R, GUARINI A, GANSBACHER B: **IL-2 treatment for cancer: from biology to gene therapy.** *Br. J. Cancer* (1992) **66**:992-998.
A detailed review on IL-2 and its application for tumour vaccination.

197. DALGLEISH AG: **The role of IL-2 in gene therapy.** *Gene Ther.* (1994) **1**:83-87.

198. WALLACH D, FELLOUS M, REVEL M: **Preferential effect of interferon-γ on the synthesis of HLA-antigens and their mRNAs in human cells.** *Nature* (1982) **299**:833-836.

199. CHEN LK, TOURVIEILLE B, BURNS GF, BACH FH, MATIEU-MAHUL D, SASPORTES M, BENSUSSAN A: **Interferon-γ: a cytotoxic T-lymphocyte differentiation signal.** *Eur. J. Immunol.* (1986) **16**:767-770.

200. NICHOLAS PR, SPIESS PJ, KARP SE, MULE JJ, ROSENBERG SA: **A nonimmunogenic sarcoma transduced with cDNA for interferon-γ elicits CD8+ T-cells against the wild-type tumor: correlation with antigen presentation capability.** *J. Exp. Med.* (1992) **175**:1423-1431.

201. PORGADOR A, BANNERJI R, WATANABE Y, FELDMAN M, GILBOA E, EISENBACH L: **Antimetastatic vaccination of tumor-bearing mice with two types of IFN-γ gene-inserted tumor cells.** *J. Immunol.* (1993) **150**:1458-1470.

202. ABDEL-WAHAB ZA, OSANTO S, DARROW TL, BARBER JR, VERVAERT CE, GANGAVALLI R, MCCALLISTER TJ, SEIGLER HF: **Transduction of human melanoma cells with the gamma interferon gene enhances cellular immunity.** *Cancer Gene Ther.* (1994) **1**:171-179.

203. COLLINS T, LAPIERRE, LA, FIERS W, STROMINGER JL, POBER JS: **Recombinant human tumor necrosis factor increases mRNA levels and surface expression of HLA-A, B antigens in vascular endothelial cells and dermal fibroblasts *in vitro*.** *Proc. Natl. Acad. Sci. USA* (1986) **83**:446-450.

204. CARSWELL EA, OLD LJ, KASSEL RL, GREEN S, FIORE N, WILLIAMSON B: **An endotoxin-induced serum factor that causes necrosis of tumors.** *Proc. Natl. Acad. Sci. USA* (1975) **72**:3666-3670.

205. HARANAKA K, SATOMI N, SAKURAI A: **Antitumor activity of murine tumor necrosis factor (TNF) against transplanted murine tumors and heterotransplanted human tumors in nude mice.** *Int. J. Cancer* (1984) **34**:263-267.

206. SIDHU RS, BOLLON AP: **Tumor necrosis factor activities and cancer therapy - a perspective.** *Pharmacol. Ther.* (1993) **57**:79-128.

207. ASHER AL, MULE JJ, KASID A, RESTIFO NP, SALO JC, REICHERT CM, JAFFE G, FENDLY B, KRIEGLER M, ROSENBERG SA: **Murine tumor cells transduced with the gene for tumor necrosis factor-α: Evidence for paracrine immune effects of tumor necrosis factor against tumors.** *J. Immunol.* (1991) **146**:3227-3234.

208. BLANKENSTEIN T, QIN, Z, UBERLA K, MULLER W, ROSEN H, VOLK, HD, DIAMANTSTEIN T: **Tumor suppression after tumor cell-targeted tumor necrosis factor gene transfer.** *J. Exp. Med.* (1991) **173**:1047-1052.

209. KARP SE, FARBER A, SALO JC, HWU P, JAFFE G, ASHER AL, SHILONI E, RESTIFO NR, MULE JJ, ROSENBERG SA: **Cytokine secretion by genetically modified nonimmunogenic murine fibrosarcoma.** *J. Immunol.* (1993) **150**:896-908.

210. STOTTER H, CUSTER MC, BOLTON ES, GUEDEZ L, LOTZE MT: **IL-7 induces human lymphokine-activated killer cell activity and is regulated by IL-4.** *J. Immunol.* (1991) **146**:50-155.

211. ALDERSON MR, TOUGH TW, ZIEGLER SF, GRABSTEIN KH: **Interleukin-7 induces cytokine secretion and tumoricidal activity by human peripheral blood monocytes.** *J. Exp. Med.* (1991) **173**:923-930.

212. HOCK H, DORSCH M, DIAMANTSTEIN T, BLANKENSTEIN T. **Interleukin-7 induces CD4+ T-cell-dependent tumor rejection.** *J. Exp. Med.* (1991) **174**:1291-1298.

213. MCBRIDE WH, THACKER JD, COMORA S, ECONOMOU JS, KELLY D, HOGGE D, DUBINETT SM, DOUGHERTY GJ: **Genetic modification of a murine fibrosarcoma to produce interleukin-7 stimulates host cell infiltration and tumor immunity.** *Cancer Res.* (1992) **52**:3931-3937.

214. JICHA DL, MULE JJ, ROSENBERG SA: **Interleukin-7 generates antitumor CTL against murine sarcomas with efficacy in cellular adoptive immunotherapy.** *J. Exp. Med.* (1991) **174**:1511-1515.

215. STEINMAN RM: **The dendritic cell system and its role in immunogenicity.** *Ann. Rev. Immunol.* (1991) **9**:271-296.

216. INABA K, INABA MN, ROMANI N, AYA H, STEINMAN S: **Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor.** *J. Exp. Med.* (1992) **176**:1693-1703.

217. •• DRANOFF G, JAFFEE E, LAZENBY A, GOLUMBEK P, LEVITSKY H, BROSE K, JACKSON V, HAMADA H, PARDOLL D, MULLIGAN R: **Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting antitumor immunity.** *Proc. Natl. Acad. Sci. USA* (1993) **90**:3539-3543.

A signifcant report on the *GM-CSF* gene-modified tumour vaccination and comparison with the effects of other cytokine genes.

218. NICOLA NA, METCALF D, MATSUMOTO M, JOHNSON GR: **Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells: identification as granulocyte colony-stimulating factor (G-CSF).** *J. Biol. Chem.* (1983) **258**:9017-9023.

219. COLOMBO MP, FERRARI G, STOPPACCIARO A, PARENZA M, RODOLFO M, MAVILIO F, PARMIANI G: **Granulocyte colony-stimulating factor gene transfer suppresses tumorigenicity of a murine adenocarcinoma *in vivo*.** *J. Exp. Med.* (1991) **173**:889-897.

220. COLOMBO MP, LOMBARDI L, STOPPACCIARO A, MELANI C, PARENZA M, BOTTAZZI B, PARMIANI G: **Granulocyte colony-stimulating factor (G-CSF) gene transduction in murine adenocarcinoma drives neutrophil-mediated tumor inhibition *in vivo*: neutrophils discriminate between G-CSF-producing and G-CSF-nonproducing tumor cells.** *J. Immunol.* (1992) **149**:113-119.

221. STOPPACCIARO A, MELANI C, PARENZA M, MASTRACCHIO A, BASSI C, BARONI C, PARMIANI G, COLOMBO MP: **Regression of an established tumor genetically modified to release granulocyte colony-stimulating factor requires granulocyte-T cell cooperation and T-cell-produced interferon-γ.** *J. Exp. Med.* (1993) **178**:151-161.

222. OSTRAND-ROSENBERG S, THAKUR A, CLEMENTS V: **Rejection of mouse sarcoma cells after transfection of MHC class II gene.** *J. Immunol.* (1990) **144**:4068-4071.

223. SCHULTZ Z, KLARNET J, GIENI R, HAYGLASS K, GREENBERG P: **The role of B-cells for *in vivo* T-cell responses to a Friend virus-induced leukemia.** *Science* (1990) **249**:921-923.

224. GREENBERG PD: **Adoptive T-cell therapy of tumors: Mechanisms operative in the recognition and elimination of tumor cells.** *Adv. Immunol.* (1990) **49**:281-355.

225. MUELLER D, JENKINS M, SCHWARTZ R: **Clonal expansion *versus* function clonal inactivation: a co-stimulatory signalling pathway determines the outcome of T-cell antigen receptor occupancy.** *Ann. Rev. Immunol.* (1989) **7**:445-480.

226. GIMMI CD, FREEMAN GJ, GRIBBEN JG, SUGITA K, FREEDMAN AS, MORIMOTO C, NADLER LM: **B-cell surface anti-**

gen B7 provides a costimulatory signal that induces T-cells to proliferate and secrete interleukin 2. *Proc. Natl. Acad. Sci. USA* (1991) **88**:6575-6579.

227. KOULOVA L, CLARK E, SHU G, DUPONT B: **The CD28 ligand B7/BB1 provides a co-stimulatory signal for alloactivation of CD4+ T-cells.** *J. Exp. Med.* (1991) **173**:759-762.

228. LINSLEY P, BRADY W, URNES M, GROSMAIRE L, DAMLE N, LEDBETTER J: **CTLA-4 is a second receptor for the B-cell activation antigen B7.** *J. Exp. Med.* (1991) **174**:561-569.

229. RAZI-WOLF Z, FREEMAN G, GALVIN F, BENACERRAF B, NADLER L, REISER H: **Expression and function of the murine B7 antigen, the major co-stimulatory molecule expressed by peritoneal exudate cells.** *Proc. Natl. Acad. Sci. USA* (1992) **89**:4210-4214.

230. NABAVI N, FREEMAN G, GAULT A, GODFREY D, NADLER L, GLIMCHER L: **Signaling through the MHC class II cytoplasmic domain is required for antigen presentation and induces B7 expression.** *Nature* (1992) **360**:266-268.

231. HUI K, GROSVELD F, FESTENSTEIN H: **Rejection of transplantable AKR leukaemia cells following MHC DNA-mediated cell transformation.** *Nature* (1984) **311**:750-752.

232. WALLICH R, BULBUC N, HAMMERLING G, KATZAV S, SEGAL S, FELDMAN M: **Abrogation of metastatic properties of tumor cells by *de novo* expression of H-2K antigens following H-2 gene transfection.** *Nature* (1985) **315**:301-305.

233. FREEMAN GJ, FREEDMAN AS, SEGIL JM, LEE G, WHITMAN JF, NADLER LM: **B7, a new member of the Ig superfamily with the unique expression on activated and neoplastic B-cells.** *J. Immunol.* (1989) **143**:2714-2722.

234. FREEMAN GJ, GRIBBEN JG, BOUSSIOTIS VA, NG JW, RESTIVO VA, LOMBARD LA, GRAY GS, NADLER LM: **Cloning of B7-2: a CTLA4 counter-receptor that costimulates human T-cell proliferation.** *Science* (1993) **262**:909-911.

235. • CHEN L, ASHE S, BRADY W, HELLSTROM I, HELLSTROM KE, LEDBETTER J, MCGOWAN P, LINSLEY P: **Co-stimulation of antitumor immunity by the B7 counter-receptor for the T-lymphocyte molecules CD28 and CTLA-4.** *Cell* (1992) **71**:1093-1102.

A significant study of the B7 co-stimulatory effect on antitumour immunity.

236. • TOWNSEND S, ALLISON J: **Tumor rejection after direct co-stimulation of CD8+ T-cells by B7-transfected melanoma cells.** *Science* (1993) **259**:368-370.

A significant study of the *B7* gene-modified tumour vaccine.

237. BASKAR S, NABAVI N, GILMCHER LH, OSTRAND-ROSENBERG S: **Tumor cells expressing major histocompatibility complex class II and B7 activation molecules stimulate potent tumor-specific immunity.** *J. Immunotherapy* (1993) **14**:209-215.

238. BASKAR S, OSTRAND-ROSENBERG S, NABAVI N, NADLER L, GILMCHER LH: **Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated MHC class II molecules.** *Proc. Natl. Acad. Sci. USA* (1993) **90**:5687-5690.

239. FUJIWARA T, GRIMM EA, MUKHOPADHYAY T, ZHANG WW, OWEN-SCHAUB LB, ROTH JA: **Induction of chemosensitivity in human lung cancer cells *in vivo* by adenovirus-mediated transfer of the wild-type p53 gene.** *Cancer Res.* (1994) **54**:2287-2291.

240. LOWE SW, SCHMITT EM, SMITH SW, OSBORNE BA, JACKS T: **p53 is required for radiation-induced apoptosis in mouse thymocytes.** *Nature* (1993) **362**:847-849.

241. HU G, LIU W, HANANIA EG, FU S, WANG T, DEISSEROTH AB: **Suppression of tumorigenesis by transcription units expressing the antisense E6 and E7 messenger RNA (mRNA) for the transforming proteins of human papilloma virus and the sense mRNA for the retinoblastoma gene in cervical carcinoma cells.** *Cancer Gene Ther.* (1995) **2**:19-32.

242. OHIRA T, OHE Y, HEIKE Y, PODACK ER, OLSEN KJ, NISHIO K, NISHIO M, MIYAHARA Y, FUNAYAMA Y, OGASAWARA H, ARIOKA H, KATO H, SAIJO N: **Gene therapy for Lewis lung carcinoma with tumor necrosis factor and interleukin 2 cDNAs co-transfected subline.** *Gene Ther.* (1994) **1**:269-275.

243. CASTLEDEN S, HELSON JA, CHONG H, HART I, VILE RG: **The use of combination gene therapies for the treatment of cancer.** *J. Cell. Biochem.* (1995) **21**(Suppl. A):418.

244. CHEN SH, CHEN XH, KOSAIL KI, WANG Y, FINEGOLD MJ, RICH SS, WOO SLC: **Combination suicide and cytokine gene therapy for metastatic colon carcinoma *in vivo*.** *J. Cell. Biochem.* (1995) **21**(Suppl. A):419.

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2. The Role of Chemotherapy in Multimodality Therapy

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The role of chemotherapy in adult soft-tissue sarcomas is controversial. In this review, the author examines the effectiveness of single-agent and combination chemotherapy to manage advanced disease and the role of adjuvant combination chemotherapy to control primary tumours and prevent the spread of the disease.

Doxorubicin (Adriamycin) is still considered the most effective single agent for advanced soft-tissue sarcoma. Ifosfamide has given similar results and may have greater potential in combination therapy than cyclophosphamide. A current study using trimetrexate shows early positive results.

Doxorubicin, cyclophosphamide, vincristine and dacarbazine is currently the most efficacious combination.

The efficacy of adjuvant chemotherapy remains to be established. The majority of studies indicate some benefit with chemotherapy, particularly in the relapse-free survival rate, but no consistent improvement in overall survival has been noted.

Le rôle de la chimiothérapie dans le sarcome des tissus mous de l'adulte est discutable. Cette revue porte sur l'efficacité de la mono et de la polychimiothérapie dans le traitement des cancers à un stade avancé et du rôle de la polychimiothérapie adjuvante dans la maîtrise des tumeurs primitives et dans la prévention de la dissémination de la maladie.

La doxorubicine (Adriamycine) est toujours considérée comme le médicament qui, utilisé seul, est le plus actif contre les sarcomes des tissus mous au stade avancé. L'ifosfamide a donné des résultats comparables à ceux de la doxorubicine, et semble présenter de plus grandes possibilités en polychimiothérapie que la cyclophosphamide. Une étude présentement en cours avec le trimetrexate laisse prévoir des résultats positifs.

Actuellement, la cyclophosphamide, la vincristine et la dacarbazine représentent l'association la plus active.

L'intérêt de la chimiothérapie adjuvante reste à être démontré. La majorité des études indique qu'elle peut être d'un certain apport, particulièrement en ce qui a trait à la survie sans récidive, mais aucune amélioration notable dans la taux de survie n'a été noté.

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Although chemotherapy is of proven benefit in embryonal rhabdomyosarcomas of childhood its role in adult soft-tissue sarcomas is more controversial. This review examines the activity of single and combination cytotoxic drugs in advanced disease. The potential contribution of adjuvant chemotherapy in providing local control of primary tumours and preventing metastasis is analysed.

Advanced Disease

Single Agents

Of the anthracyclines, doxorubicin (Adriamycin [ADR]), introduced into clinical practice in the early 1970s, is generally acknowledged as the most active single agent in the treatment of adult soft-tissue sarcomas. A review of relevant studies containing more than 25 patients,[1-8] showed an average response rate of 24% (range from 16% to 41%) in 930 patients. An intensive search to identify anthracyclines that retain the antitumour efficacy of ADR with less toxicity, especially cardiotoxicity, has been disappointing; Carminomycin,[9] deoxydoxorubicin,[10] Aclacinomycin[11,12] and demethoxydaunorubicin,[13] each showed insignificant activity. When given at identical doses (75 mg/m^2 every 3 weeks) in a study performed by the European Organisation for Re

search and Treatment of Cancer (EORTC), Epiadriamycin was less toxic than ADR, but the response rate was lower — 15 of 84 (18%) versus 21 of 83 (25%) for ADR.[14] This difference was not statistically significant, but it is unlikely that Epiadriamycin, even if used at a dose equitoxic to ADR, will be more active.

With respect to alkylating agents, ifosfamide, an interesting analogue of cyclophosphamide, was synthesized in Germany in 1967. Reported response rates are available on 218 patients treated with a variety of doses and schedules of ifosfamide.[15] The average rate of 28% (range from 18% to 38%) is remarkably similar to that for ADR.[16] However ifosfamide has been evaluated in considerably fewer patients, and characteristically the response rate of a new drug is inversely proportional to the number of patients in which it is evaluated. Nevertheless, ifosfamide, in contrast to ADR, has been used more frequently as second-line chemotherapy. Response rates in an EORTC study comparing ifosfamide with cyclophosphamide were 18% and 8% respectively[17] and provided the only single-agent data for cyclophosphamide. Ifosfamide produced two responses in 28 previously treated patients and three responses in 31 patients crossed over after failure of cyclophosphamide. The response rates for patients who had never received chemotherapy were 10 of 40 (25%) for ifosfamide and 5 of 38 (13%) for cyclophosphamide. Although the rates were not significantly different by conventional statistical analysis, ifosfamide caused less myelosuppression than cyclophosphamide, suggesting greater potential in combination.

Of other single agents, it is generally accepted that dacarbazine (DTIC) shows limited activity in soft-tissue sarcomas,[18,19] the average response rate in accumulated series (97 patients) being 18% (range from 15% to 25%). Cisplatinum is very active in osteosarcoma and has been extensively tested in soft-tissue sarcoma.[2,3,5] An average response rate of 9% in 150 patients does not warrant inclusion in combination chemotherapy, particularly as it is a very toxic and expensive drug. The data on methotrexate are intriguing. A study from the United Kingdom[20] reported a response rate of 25% in 62 patients. A variety of doses, routes and schedules were used in this trial. A later EORTC study was unable to demonstrate any activity for methotrexate given intravenously every week in previously treated patients.[21] Although high-dose methotrexate has been used in several small series,[1] there is no evidence that its activity is increased, and no group has repeated the studies of conventional-dose methotrexate. The Canadian Sarcoma Group, in conjunction with the National Cancer Institute of Canada, is currently performing a phase II study using trimetrexate, a methotrexate analogue, and responses have been seen.

Table I – Response to Combination Chemotherapy

Combination	No. of patients	Response, %
CYVADIC*	750	48 (15–68)
ADR/DTIC	592	27.5 (20–42)
CYCLO/VCR/ADR	70	19
CYCLO/ADR/DTIC	80	35
CYCLO/VCR/ADR/DACT	199	40
CYCLO/ADR/MTX	100	36

*Cyclophosphamide, vincristine, Adriamycin, dacarbazine.
ADR = Adriamycin, DTIC = dacarbazine, CYCLO = cyclophosphamide, VCR = vincristine, DACT = actinomycin D, MTX = methotrexate.

Table II – Combination Chemotherapy Including Ifosfamide

Series	Combination	No. of patients	Response, %
Wiltshaw and associates, 1986[23]	ADR + IFOS	47	36
Schutte and associates, 1986[22]	ADR + IFOS	178	36
Elias and Antman, 1986[24]	ADR + IFOS + DTIC	65	52

ADR = Adriamycin, IFOS = ifosfamide, DTIC = dacarbazine.

Combination Chemotherapy

The drug combination most commonly used to treat adult soft-tissue sarcomas is CYVADIC (Table I). It consists of cyclophosphamide (500 mg/m^2), vincristine (1.4 mg/m^2, day 1), ADR (50 mg/m^2) and dacarbazine (250 mg/m^2, days 1 to 5).[1-8] The two most active single agents (ADR and dacarbazine) have also been used fairly extensively in combination,[2,6] but the response rate (Table I) seems lower than that of CYVADIC. Other ADR combinations have activity in the same range, but none are better than CYVADIC.[1-8]

The addition of ifosfamide to ADR[22,23] has been disappointing (Table II). In the EORTC study[22] of 178 patients treated with ADR and ifosfamide, the overall response rate of 36% was similar to that reported for CYVADIC (39%). However, nearly half the patients receiving CYVADIC manifested disease progression, whereas the corresponding figure for ADR and ifosfamide was 19%. The addition of dacarbazine seemed to improve the response rate.[24] However, this particular regimen was very myelosup-

pressive; there was a 23% incidence of granulocytopenic fever. A central venous line had to be inserted in all patients and treatment was given by intravenous infusion for 5 days every 3 weeks.

The EORTC is currently comparing ADR, CYVADIC and ADR plus ifosfamide in a three-arm study; 393 patients have been entered in 22 months and the accrual should be complete within 6 months. Comparative response rates are not yet available, but should be very interesting as ADR has never been directly compared with CYVADIC. This study should also clarify the value of ifosfamide used in combination.

The Canadian Sarcoma Group is performing a pilot study of the combination of ADR, ifosfamide and dacarbazine, using a shorter, more convenient schedule than the Boston group. The Boston group is comparing their schedule of this combination with ADR alone.

Primary Management

Intra-arterial Chemotherapy

To improve local control, chemotherapy has been given intra-arterially in conjunction with surgery and radiotherapy. There have been no randomized studies comparing this route of therapy with other methods for local control, such as radical surgery or limited surgery plus radiotherapy. Investigators claiming superiority for intra-arterial therapy have generally compared their results with historical controls from their own centre or results reported in the literature, both of which have obvious flaws.

Another method of primary management has been that of isolation perfusion with or without hyperthermia. Most studies using this method were performed between 1960 and 1980 with drugs such as melphalan, actinomycin D and nitrogen mustard, whose activity in soft-tissue sarcomas is minimal. The results of several of these series[25-28] are compared in Table III with the results reported by Lindberg and colleagues[29] from a group of patients treated by surgery and radiotherapy in a major centre. Limb salvage, local recurrence and ultimate outcome in terms of overall survival, show little advantage for the complex, expensive and cumbersome technique of isolation perfusion.

The intra-arterial use of ADR with or without radiotherapy preoperatively has been reported (Table IV).[30-32] Eilber's group[33,34] has the most extensive experience and their rates of limb salvage and local control are impressive. However, complications, including pathologic fracture of long bones, led them to reduce the dose of radiotherapy in

Table III – Isolation Perfusion With Melphalan, Actinomycin D and Nitrogen Mustard Plus Hyperthermia Compared With Surgery Plus Radiotherapy

Series	No. of patients	Limb salvage, %	Local recurrence, %	Metastases, %	Overall survival, %
Chemotherapy					
Stehlin and associates, 1984[25]	65	94	NS	NS	73
Lehti and associates, 1986[26]	64	83	11	33	67
McBride, 1976[27]	85	89	15	23	68
Krementz and associates, 1977[28]	73	90	25	30	64
No chemotherapy					
Lindberg and associates, 1981[29]	200	85	20	25	69

NS = not stated.

Table IV – Intra-arterial Adriamycin With Radiotherapy Compared With Preoperative and Postoperative Radiotherapy Without Chemotherapy

Series	No. of patients	Follow-up, mo.	Limb salvage, %	Local recurrence, %	Metastases, %	Overall survival, %
Chemotherapy						
Mantravadi and associates, 1984[30]	32	15	91	3	25	70
Denton and associates, 1984[31]	15	24	80	6	NS	79
Goodnight and associates, 1985[32]	17	32	88	0	35	82
Eilber and associates, 1984[33] 1985[34]						
Protocol 2	77	60	96	4	NS	64
Protocol 3	105	24	97	8	NS	95
No chemotherapy						
Karakousis and associates, 1986[35]	85	>24	96	13	NS	68
Abbatucci and associates. 1986[36]	89	60	93	14	NS	66

NS = not stated.

protocol 3 (from 3500 rad in 10 fractions to 1750 rad) and this doubled the local recurrence rate. Judging from the results of protocol 2, the rate of metastasis was not affected by improved local control and the survival rate may not be better. Protocol 3 still has a very short follow-up. When the results of preoperative and postoperative irradiation as in the studies by Karakousis and associates[35] and Abbutucci and colleagues,[36] are used for comparison (Table IV), the local recurrence rates with radiotherapy only are slightly higher, but the outcome is otherwise similar, again casting doubt on the value of intra-arterial chemotherapy. Eilber's group is currently conducting a randomized trial comparing intra-arterial versus intravenous preoperative chemotherapy, and it may be that the theoretical advantages of intra-arterial chemotherapy will not be clinically evident.

Systemic Adjuvant Chemotherapy

Since the frequency of soft-tissue sarcoma is approximately 2 in 100 000, the difficulties of performing good randomized studies of adjuvant chemotherapy in this heterogeneous group of tumours are obvious. Most studies published or in progress are flawed.

To examine the role of adjuvant ADR, the results of five randomized studies[37-41] are summarized (Table V); each of them compares a chemotherapy group with a randomized control group treated only by surgery with or without radiotherapy. The Scandinavian study of Alvegard[40] is the largest but has been reported only in abstract form and the follow-up is relatively short. Nevertheless, it has the greatest power to detect differences between the two arms, and the various prognostic factors are more likely to be evenly distributed. The Boston/ECOG study of Wilson and colleagues[41] has much longer follow-up than the other studies, and although there was a slight delay in the appearance of metastases in the ADR arm, there have been no significant differences in disease-free survival or overall survival for all patients or those with extremity sarcomas only.

The only study giving positive results, that of Gherlinzoni and colleagues from Bologna,[37] has been severely criticized.[42] The number of patients was relatively small and there were three different surgical groups. These factors, combined with a poor randomization technique, led to considerable imbalance in prognostic factors between the arms. A recent update of this study,[43] involving 77 patients (33 receiving ADR and 44 controls), demonstrated improvement in relapse-free and overall survival for the ADR treated group, but the median duration of follow-up was not stated.

At present there is no firm evidence that adjuvant single-agent ADR affords any substantial benefit in soft-tissue sarcomas. However, delay in the appearance of metastases in some studies suggests that more aggressive chemotherapy may be worth pursuing.

Randomized studies of various types of combination chemotherapy versus control are depicted in Table VI.[44-48]

The first study was performed by Lindberg and colleagues at the

Table V – Randomized Studies of Adjuvant Adriamycin

Series	No. of patients	Median follow-up, mo	Outcome
Gherlinzoni and associates, 1986[37]	59	28	RFS $p < 0.005$ in favour of ADR. No data on overall survival
Antman and associates, 1987[38]	64	20	NS
Eilber and associates, 1986[39]	114	30	NS
Alvegard, 1986[40]	146	36	NS
Wilson and associates, 1986[41]	75	49	NS

RFS = relapse-free survival, NS = not significant.

Table VI – Randomized Studies of Adjuvant Combination Chemotherapy

Series	Combination	No. of patients	Median follow-up, mo	Outcome
Edmonson and associates (Mayo Clinic), 1984[44]	VCR/DACT/CYCLO alternating VCR/ADR/DTIC 12 mo	61	64	NS
Lindberg and associates (M.D. Anderson Hospital), 1976[45]	VCR/ADR/CYCLO	43	120	RFS, $p = 0.04$ in favour of chemotherapy OS NS
Benjamin and associates (M.D. Anderson Hospital), 1987[46]	VCR/DACT/CYCLO 24 mo			
Rosenberg and associates (National Cancer Institute), 1985[47]	ADR/CYCLO/HDMTX 14 mo	65	60	RFS and OS, $p = 0.04$ in favour of chemotherapy
Bramwell and associates, 1987[48]	CYVADIC 8 mo	317	37	RFS, $p = 0.01$ in favour of chemotherapy OS NS

NS = not significant, OS = overall survival, HDMTX = high-dose methotrexate.

M.D. Anderson Hospital and Tumor Institute in the early 1970s and reported after 18 months' follow-up.[45] At that time, the chemotherapy group was doing slightly, although not significantly, worse. Recently this study has been reanalysed after 10 years' follow-up.[46] The relapse-free survival now favours the chemotherapy group, but although overall survival is better for that group, the difference is not significant. Local recurrence was lower in the chemotherapy arm (two versus eight), but metastases occurred with similar frequency — 45% (9 of 20) in the chemotherapy group versus 48% (11 of 23) in controls.

Because the early results of the M.D. Anderson Hospital study were negative, the trial from the United States National Cancer Institute was the first randomized study[47] to suggest benefit for adjuvant chemotherapy. It should be emphasized that this study had serious flaws — the number of patients was very small, increasing the likelihood of imbalance of known and, more importantly, unknown prognostic factors between the arms; the control group has done worse than expected when compared with results reported by other centres, thus magnifying the difference between the two arms. Because the ADR dose was pushed to tolerance there was a 30% incidence of subclinical cardiomyopathy detected by nuclear medicine scans, and three patients suffered congestive heart failure. Benefit was observed only in extremity sarcomas; the same chemotherapy had no effect in head, neck and trunk sarcomas. The most recent analysis,[49] with median follow-up of 7 years, no longer demonstrates improved survival for the chemotherapy group ($p = 0.124$).

Building on the results of their previous study,[47] and dismayed by the high incidence of cardiomyopathy, Rosenberg and colleagues compared their "standard" chemotherapy with an abbreviated course of treatment, giving much lower total doses of ADR and cyclophosphamide and omitting high-dose methotrexate.[49] There were no significant differences between the two treatments, in terms of 5-year disease-free (72%) and overall (75%) survival. In view of the lack of efficacy of ADR as an adjuvant, and the minimal activity of cyclophosphamide in advanced disease, it would be surprising if this combination proves to be effective.

A Mayo Clinic study[44] did not demonstrate any benefit for adjuvant chemotherapy, but the incidence of local recurrence (30%) was high in this trial, perhaps because radiotherapy was not used. In addition, the chemotherapy used was inadequate — the vincristine, actinomycin D, cyclosphosphamide combination is inactive in advanced disease, and as vincristine, ADR, dacarbazine was only alternated every 6 weeks with the first combination, patients received active drugs only every 12 weeks, and at a low dose intensity.

For all patients in the EORTC study,[48] there was a significant improvement in relapse-free survival in the CYVADIC arm ($p = 0.01$), although this is of borderline significance on subgroup analysis by site, for both limb sarcomas ($p = 0.06$) and those of the head, neck and trunk ($p = 0.09$). However, there was no benefit for any group in terms of overall survival. In fact, the improvement in relapse-free survival was entirely accounted for by reduced local recurrence in the CYVADIC arm ($p = 0.005$) whereas distant metastases occurred with equal frequency in both arms ($p = 0.28$). It is intriguing that the reduction in local recurrence seems to be occurring in head, neck and trunk tumours.

Conclusions

Although the majority of studies suggest some limited benefit for adjuvant chemotherapy, the optimal regimen and timing of such therapy remain to be established. The conclusions of the National Institutes of Health Consensus Development Conference on Bone and Soft-Tissue Sarcomas, 1984, remain relevant — "the efficacy of adjuvant systemic chemotherapy for high grade sarcomas remains to be established within the context of prospective clinical trials."[50]

References

1. Bramwell VHC, Pinedo HM: Bone and soft tissue sarcomas. In Pinedo HM (ed): *Cancer Chemotherapy; the EORTC Cancer Chemotherapy Annual*, Excerpta Medica, Amsterdam, 1979: 424–450
2. Idem: Bone and soft tissue sarcomas. In Pinedo HM (ed): *Cancer Chemotherapy; the EORTC Cancer Chemotherapy Annual*, Excerpta Medica, Amsterdam, 1980: 393–414
3. Idem: Bone and soft tissue sarcomas. In Pinedo HM (ed): *Cancer Chemotherapy; the EORTC Cancer Chemotherapy Annual*, Excerpta Medica, Amsterdam, 1981: 409–424
4. Bonadonna G, Santoro A: Bone and soft tissue sarcomas. In Pinedo HM (ed): *Cancer Chemotherapy; the EORTC Cancer Chemotherapy Annual*, Excerpta Medica, Amsterdam, 1982: 373–396
5. Idem: Bone and soft tissue sarcomas. In Pinedo HM, Chabner BA (eds): *Cancer Chemotherapy; the EORTC Cancer Chemotherapy Annual*, Excerpta Medica, Amsterdam, 1983: 430–445
6. Idem: Bone and soft tissue sarcomas. In Pinedo HM, Chabner BA (eds): *Cancer Chemotherapy; the EORTC Cancer Chemotherapy Annual*, Excerpta Medica, Amsterdam, 1984: 436–449
7. Santoro A, Bonadonna G: Soft tissue and bone sarcomas. In Pinedo HM, Chabner BA (eds): *Cancer Chemotherapy; the EORTC Cancer Chemotherapy Annual*, Excerpta Medica, Amsterdam, 1985: 426–438
8. Idem: Soft tissue and bone sarcomas. In Pinedo HM, Chabner BA (eds): *Cancer Chemotherapy; the EORTC Cancer Chemotherapy Annual*, Excerpta Medica, Amsterdam, 1986: 495–510.
9. Bramwell VH, Mouridsen HT, Mulder JH, et al: Carminomycin vs adriamycin

in advanced soft tissue sarcomas: an EORTC randomised phase II study. *Eur J Cancer Clin Oncol* 1983; 19: 1097–1104

10. RAYMOND V, MACILL GB, WISSEL PS, et al: Phase II trial of deoxydoxorubicin (DXDX) in patients (pts) with soft tissue sarcoma (STS) (abstr). *Proc Am Soc Clin Oncol* 1986; 5: 146
11. BORDEN E, AMATO D, EARHART R, et al: Phase II evaluation of diazonoleucine (DON) and Aclacinomycin (ACM-A) for soft tissue or bone sarcomas and mesothelioma (abstr). *Ibid:* 131
12. BERTRAND M, MULTHAUF P, BARTOLUCCI A, et al: Phase II study of aclarubicin in previously untreated patients with advanced soft tissue sarcoma: a Southeastern Cancer Study Group trial. *Cancer Treat Rep* 1985; 69: 725–726
13. RAYMOND V, MACILL GB, WELT S, et al: 4-demethoxydaunorubicin (DMDR) in advanced soft tissue sarcomas (STS): A phase II study (abstr). *Proc Am Soc Clin Oncol* 1983; 2: 235
14. MOURIDSEN HT, BASTHOLT L, SOMERS R, et al: Adriamycin versus epirubicin in advanced soft tissue sarcomas. A randomized phase II/phase III study of the EORTC Soft Tissue and Bone Sarcoma Group. *Eur J Cancer Clin Oncol* 1987; 23: 1477–1483
15. BRADE WP, HERDRICH K, VARINI M: Ifosfamide — pharmacology, safety and therapeutic potential. *Cancer Treat Rev* 1985; 12: 1–47
16. SAROSY G, LIONETTO R, BRAMWELL VHC, et al: Ifosfamide: an old drug with a bright future. *JAMA* (in press)
17. BRAMWELL VH, MOURIDSEN HT, SANTORO A, et al: Cyclophosphamide versus ifosfamide: final report of a randomized phase II trial in adult soft tissue sarcomas. *Eur J Cancer Clin Oncol* 1987; 23: 311–321
18. GOTTLIEB JA, BENJAMIN RS, BAKER LH, et al: Role of DTIC (NSC-45388) in the chemotherapy of sarcomas. *Cancer Treat Rep* 1976; 60: 199–203
19. BUESA JM, MOURIDSEN H, VAN OOSTEROM AT, et al: High-dose DTIC in advanced soft tissue sarcomas (STS) of the adult. A phase II study of the EORTC Soft Tissue and Bone Sarcoma Group (abstr). 5th NCI-EORTC Symposium on New Drugs in Cancer Therapy, October 22–24, 1986, Amsterdam
0. WILTSHAW E, HARMER C, MCKINNA A: Soft tissue sarcoma: treatment of advanced disease in the Royal Marsden Hospital. Paper presented at the International Course on Recent Advances in the Treatment of Ovarian and Testicular Cancer and of Soft Tissue and Bone Sarcomas, Noordwijkerhort, Netherlands, December 6–8, 1979

BUESA JM, MOURIDSEN HT, SANTORO A, et al: Treatment of advanced soft tissue sarcomas with low-dose methotrexate: a phase II trial by the European Organization of Research on Treatment for Cancer (EORTC) Soft Tissue and Bone Sarcoma Group. *Cancer Treat Rep* 1984; 68: 683–684

22. SCHUTTE J, DOMBERNOWSKY P, SANTORO A, et al: Adriamycin (A) and Ifosfamide (I), a new effective combination in advanced soft tissue sarcoma (abstr). *Proc Am Soc Clin Oncol* 1986; 5: 145
23. WILTSHAW E, WESTBURY G, HARMER C, et al: Ifosfamide plus mesna with and without adriamycin in soft tissue sarcoma. *Cancer Chemother Pharmacol* 1986; 18 (suppl 2): S10–12
24. ELIAS AD, ANTMAN KH: Doxorubicin, ifosfamide, and dacarbazine (AID) with mesna uroprotection for advanced untreated sarcoma: a phase I study. *Cancer Treat Rep* 1986; 70: 827–833
25. STEHLIN JS JR, GIOVANELLA BC, GUTIERREZ AE, et al: 15 years' experience with hyperthermic perfusion for treatment of soft tissue sarcoma and malignant melanoma of the extremities. *Front Radiat Ther Oncol* 1984; 18: 177–182
26. LEHTI PM, MOSELEY HS, JANOFF K, et al: Improved survival for soft tissue sarcoma of the extremities by regional hyperthermic perfusion, local excision and radiation therapy. *Surg Gynecol Obstet* 1986; 162: 149–152
27. MCBRIDE CM: Regional chemotherapy for soft tissue sarcomas. In: *Management of Primary Bone and Soft Tissue Tumors*, Year Bk Med, Chicago, 1977: 353–360
28. KREMENTZ ET, CARTER RD, SUTHERLAND CM, et al: Chemotherapy of sarcomas of the limbs by regional perfusion. *Ann Surg* 1977; 185: 555–564
29. LINDBERG RD, MARTIN RG, ROMSDAHL MM, et al: Conservative surgery and postoperative radiotherapy in 300 adults with soft-tissue sarcomas. *Cancer* 1981; 47: 2391–2397
30. MANTRAVADI RV, TRIPPON MJ, PATEL MK, et al: Limb salvage in extremity soft-tissue sarcoma: combined modality therapy. *Radiology* 1984; 152: 523–526
31. DENTON JW, DUNHAM WK, SALTER M, et al: Preoperative regional chemotherapy and rapid-fraction irradiation for sarcomas of the soft tissue and bone. *Surg Gynecol Obstet* 1984; 158: 545–551
32. GOODNIGHT JE JR, BARGAR WL, VOEGELI T, et al: Limb-sparing surgery for extremity sarcomas after preoperative intraarterial doxorubicin and radiation therapy. *Am J Surg* 1985; 150: 109–113
33. EILBER FR, MIRRA J, ECKARDT J, et al: Intraarterial Adriamycin, radiation therapy, and surgical excision for extremity skeletal and soft tissue sarcomas. *Dev Oncol* 1984; 26: 141–152
34. EILBER FR, GUILIANO AE, HUTH J, et al: Limb salvage for high-grade soft tissue sarcomas of the extremity: experience at the University of California. In U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health: *Cancer Treatment Symposia*, The Institutes, Bethesda, Md., 1985: 49–58
35. KARAKOUSIS CP, EMRICH LJ, RAO U, et al: Feasibility of limb salvage and survival in soft tissue sarcomas. *Cancer* 1986; 57: 484–491
36. ABBATUCCI JS, BOULIER N, DE RANIERI J, et al: Local control and survival in soft tissue sarcomas of the limbs, trunk walls and head and neck: a study of 113 cases. *Int J Radiat Oncol Biol Phys* 1986; 12: 579–586
37. GHERLINZONI F, BACCI G, PICCI P, et al: A randomized trial for the treatment of high-grade soft-tissue sarcomas of the extremities: preliminary observations. *J Clin Oncol* 1986; 4: 552–558
38. ANTMAN K, AMATO D, PILEPICH M, et al: A randomized intergroup trial of adjuvant doxorubicin (DOX) for soft tissue sarcomas (STS): lack of apparent differences between treatment groups (abstr). *Proc Am Soc Clin Oncol* 1987; 6: 134
39. EILBER FR, GIULIANO AE, HUTH JF, et al: Adjuvant adriamycin in high grade extremity soft tissue sarcoma — a randomized prospective trial (abstr). *Proc Am Soc Clin Oncol* 1986; 5: 125
40. ALVEGARD TA: Adjuvant chemotherapy with adriamycin in high grade malignant soft tissue sarcoma — a Scandinavian randomized study (abstr). *Ibid:* 125
41. WILSON RE, WOOD WC, LERNER HL, et al: Doxorubicin chemotherapy in the treatment of soft-tissue sarcoma. Combined results of two randomized trials. *Arch Surg* 1986; 121: 1354–1359
42. SYLVESTER R: Soft-tissue sarcomas of the extremities (C). *J Clin Oncol* 1987; 5: 321–322
43. PICCI P, BACCI G, GHERLINZONI F, et al: Results of a randomized trial for the treatment of localized soft tissue tumors (STS) of the extremities in adult patients. In RYAN JR, BAKER LH (eds): *Recent Concepts in Sarcoma Treatment: Proceedings of the International Symposium on Sarcomas, Tarpon Springs, Florida, October 8–10, 1987*, Kluwer Academic, Dordrecht, The Netherlands, 1988: 144–148
44. EDMONSON JH, FLEMING TR, IVINS JC, et al: Randomized study of systemic chemotherapy following complete excision of nonosseous sarcomas. *J Clin Oncol* 1984; 2: 1390–1396
45. LINDBERG RD, MURPHY UK, BENJAMIN RS, et al: Adjuvant chemotherapy in the treatment of primary soft tissue sarcomas: a preliminary report. In: *Management of Primary Bone and Soft Tissue Tumors*, Year Bk Med, Chicago, 1977: 343–352
46. BENJAMIN RS, TERJANIAN TO, FENDGLIO CJ, et al: The importance of combina-

tion chemotherapy for adjuvant treatment of high risk patients with soft tissue sarcomas of the extremities. In SALMON SE (ed): *Adjuvant Therapy of Cancer V*, Grune, New York, 1987: 735-744

47. ROSENBERG SA, CHANG AE, GLATSTEIN E: Adjuvant chemotherapy for treatment of extremity soft tissue sarcomas: review of the National Cancer Institute experience. In U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health: *Cancer Treatment Symposia*, The Institutes, Bethesda, Md., 1985: 83-88
48. BRAMWELL VHC, ROUESSE J, STEWARD W, et al: European experience of adjuvant chemotherapy for soft tissue sarcoma: an interim report of a randomized trial of CYVADIC vs control. In RYAN JR, BAKER LH (eds): *Recent Concepts in Sarcoma Treatment: Proceedings of the International Symposium on Sarcomas, Tarpon Springs, Florida, October 8-10, 1987*, Kluwer Academic, Dordrecht, Netherlands, 1988: 157-164
49. BAKER AR, CHANG AE, GLATSTEIN E al: National Cancer Institute experie in adjuvant chemotherapy for the m agement of patients with high gr extremity soft tissue sarcomas. In *I.* 123-130
50. LAWRENCE W: Consensus conferer Limb sparing treatment of adult s tissue sarcomas and osteosarcon *JAMA* 1985; 254: 1791-1794

SESAP V Question

Items 253-255

A 54-year-old man has noted an increase in the size of his left thigh over the last 18 months. Physical examination reveals a fairly well-circumscribed, rubbery, nontender, 9.0- × 5.0-cm mass on the medial aspect of the thigh, noticeably more prominent with the knee extended. The patient has no history of trauma or recent infection.

253. The procedure LEAST likely to aid in management of this patient is

(A) conventional roentgenography of the thigh

(B) angiography

(C) ultrasonography of the thigh

(D) computed tomography of the thigh

(E) lymphangiography

254. A soft tissue mass is demonstrated in a subfascial plane of the thigh. The most appropriate method for determining the histopathology would be

(A) multiple percutaneous aspiration needle biopsies

(B) incisional biopsy

(C) "core" needle biopsy

(D) enucleation of the lesion for biopsy

(E) wide local excision

255. Pathologic examination shows liposarcoma. Definitive treatment might include each of the following EXCEPT

(A) wide anatomic soft-part resection

(B) adjuvant postoperative radiotherapy

(C) adjuvant postoperative chemotherapy

(D) preoperative radiation therapy

(E) preoperative systemic chemotherapy

For the incomplete statements above, select the one completion for each that is best of the five given.

For the critique of Items 253-255 see page 426.

Growth Suppression Mediated by Transfection of p53 in Hut292DM Human Lung Cancer Cells Expressing Endogenous Wild-Type p53 Protein

C57

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Abstract

This study was undertaken to analyze the effect of wild-type p53 transfection on the growth potential of a human lung cancer cell line Hut292DM expressing endogenous wild-type p53. Transfection efficiencies obtained with either the wild-type or a mutant p53 complementary DNA revealed a significant decrease in the number of colonies obtained with the wild-type p53 as compared to the mutant p53 complementary DNA (27%) or control vector DNA only (20%), suggesting that wild-type p53 inhibited the growth of Hut292DM cells. A series of wild-type and mutant p53 transfection clones were then analyzed for the presence and expression of the exogenous *p53* gene. Polymerase chain reaction amplification revealed that 98% of mutant p53 transfection clones analyzed contained the exogenous *p53* gene as opposed to 47% for wild-type p53 clones. The majority of mutant p53 clones expressed high levels of exogenous p53 mRNA and protein as analyzed by Northern and Western blots, respectively. In contrast, all wild-type p53 clones analyzed failed to express exogenous p53 mRNA transcript or protein of a normal size. Aberrant-size p53 mRNA was detected in two wild-type p53 clones (X833.W2 and W18), and Western blot analysis revealed that these clones expressed truncated p53 proteins (M_r 45,000 and 33,000 respectively). No difference in proliferation rates *in vitro* or in tumorigenic potential in nude mice were observed between mutant p53 clones or control cell lines. In contrast, a wild-type p53 clone (X833.W2) exhibited a significantly reduced tumorigenic potential in nude mice, whereas its *in vitro* proliferation rate was comparable to parental Hut292DM cells. The data indicate that exogenous expression of wild-type p53 is incompatible with Hut292DM lung cancer cell proliferation *in vitro* and suggest that p53-mediated growth control *in vitro* and *in vivo* may be dissociated and exerted by separate domains of the p53 protein.

Introduction

Since its discovery approximately a decade ago, the *p53* tumor suppressor gene has attracted a great deal of attention in cancer research, partly due to its capacity to prevent uncontrolled growth of cancer cells and due to the realization that most types of human cancers studied, including lung, display some form of alteration of the *p53* gene (1). The molecular mechanisms by which *p53* may regulate cell cycle progression and therefore prevent tumor cell growth are poorly resolved at the present time. A variety of human tumor cells have now been shown to be growth arrested when transfected with the *p53* tumor suppressor gene (2–5), and the cell cycle progression of these recipient cells is blocked in the G_1-S transition phase (3, 6). However, this effect is seen only when the cancer cells contain mutated p53 or are null for expression. To date, reports of the introduction of wild-type p53 cDNA[2] into tumor cells containing endogenous wild-type p53 have shown that there is no effect on *in vitro* growth (2, 4).

The product of the *p53* gene is a 393-amino-acid-long protein, primarily localized in the cell nucleus, where it may interact with genomic DNA sequences to regulate the cell cycle in two ways. The carboxy-terminal portion of the protein is associated with DNA binding, and a consensus DNA sequence which binds to p53 has now been defined that shares homology with DNA origins of replication (7, 8). The amino-terminal domain functions as a transcriptional modulator, possibly involved in the repression of nuclear oncogene transcription (9). In addition, p53 may control cell growth by binding to, and interacting with, nuclear proteins critically involved in cell cycle regulation such as the cyclin-dependent cdc2 kinase (10) or the murine double minute 2 oncogene product (11). Recent studies have shown that many cancer cells no longer express a normal p53 protein, the result of both alleles being inactivated by a combination of single allele deletion and point mutation of the remaining allele (12, 13). However, a significant proportion of human cancers arise in which no detectable alteration in p53 expression is observed, seemingly precluding any role of the *p53* tumor suppressor gene in the natural development of these tumors. The present study was undertaken to determine whether human lung cancer cell growth could be influenced by transfection of the *p53* gene. To our knowledge, we are the first to report growth suppression induced by high-level expression of exogenous wild-type p53 in lung cancer cells expressing normal endogenous p53 protein.

Materials and Methods

Transfection. Human lung cancer Hut292DM cells were kindly provided by C. Harris (14) and were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. Transfections were performed by the polybrene-dimethyl sulfoxide technique as described (15) using plasmid constructs obtained from B. Vogelstein: pCMVneoBam, a eukaryotic expression vector containing the cytomegalovirus constitutive promoter and the neomycin resistance gene under control of the simian virus 40 promoter; pC53SN3, derived from pCMVneo by insertion of a full-length human wild-type p53 cDNA under control of the cytomegalovirus promoter; and pC53SCX3, which contains a single point mutation in the p53 cDNA at codon 143 (substitution of alanine to valine). Hut292DM cells (2×10^6) were transfected with 20 μg of plasmid DNA, and following 3–4 weeks of selection in medium containing 1100 μg/ml geneticin, colonies were either stained with 5% Giemsa to assess transfection efficiency or cloned and expanded for further analysis.

Molecular Analysis of p53 Expression. Standard nucleic acid procedures were performed as described (15). PCR analysis was performed

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[2] The abbreviations used are: cDNA, complementary DNA; PCR, polymerase chain reaction.

using DNA extracts obtained by the rapid detergent method described (4). The two primers used for p53 cDNA amplification (p53.1: 5'-CAC GAC GGT GAC ACG CTT CCC TG-3'; p53.2: 5'-GTC CTG GGT GCT TCT GAC GCA CAC-3') are complementary to 5' and 3' flanking sequences of the p53 coding region, respectively, and yield a 1.2-kilobase PCR product. Southern and Northern blot analyses were performed using genomic DNA extracts digested with *Bam*HI or total cytoplasmic RNA extracted as described (16). Nucleic acids were separated on 1% agarose gels (DNA) or 1.2% agarose-formaldehyde denaturing gels (RNA) and transferred to nitrocellulose membranes. All hybridizations were performed using a 1.8-kilobase full-length p53 cDNA probe derived from pC53SN (following *Bam*HI digestion) and radiolabeled by the random primer method. Protein extraction and Western blot analysis were performed as described (17). Protein extracts were isolated from 50% confluent cells using RIPA buffer for cell lysis, and the protein concentration was determined by a modified Lowry colorimetric assay (DC protein assay kit; BioRad, Richmond, CA). Equal amounts of protein extracts (100 μg) were denatured (5 min at 100°C in presence of 0.1 M dithiothreitol), separated through 10% polyacrylamide-sodium dodecyl sulfate gels, and transferred to nitrocellulose membranes. Immunoblotting was performed using the monoclonal antibody PAb 1801 (Oncogene Science, Manhasset, NY) diluted 1:500 as the primary antibody and a horseradish peroxidase-conjugated goat anti-mouse IgG (BioRad) diluted 1:2000 as the secondary antibody. Detection of p53 antigen was performed using an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) and following the manufacturer's recommendations.

Cell Growth Analysis and Tumorigenicity Assay. Cells (1.5×10^5) were plated in 35-mm tissue culture wells, and at various time points cells from duplicate wells were counted. Culture medium was changed 3 times each week. Tumorigenicity was assessed by inoculating 5×10^6 cells at 6 sites into 4–6-week-old athymic nude mice (one inoculation per animal). Tumors were measured with linear calipers at regular intervals to compare growth properties between the different cell lines.

Results and Discussion

Three separate transfection experiments were performed in an attempt to express exogenous p53 cDNA (wild-type and mutant versions) in Hut292DM human lung cancer cells. Transfection efficiencies (see Table 1) from all three experiments indicate a significant decrease in the number of geneticin-resistant colonies obtained with the wild-type p53 cDNA as compared to the vector construct alone. In contrast, the mutant p53 cDNA did not appear to affect the transfection efficiency. These results suggested that expression of exogenous wild-type p53 inhibits Hut292DM cell growth or that high levels of expression are incompatible with sustained proliferation of these cells. A detailed molecular analysis of p53 expression in a series of transfection clones was then undertaken to gain more insight into the possible role of p53 in the control of Hut292DM tumor cell growth. PCR analysis of p53 cDNA was performed on a series of mutant and wild-type p53 transfection clones derived from two separate experiments (X813 and X833). The majority of X833 mutant p53 clones analyzed displayed a 1.2-kilobase PCR amplification product which contains the entire p53 coding region (16 of 18, or 89%) whereas only 47% (8 of 17) of the wild-type p53 transfection clones displayed a p53 cDNA-related PCR product (Fig. 1*A*). Interestingly, one of these clones (X833.W2) yielded an unusually large PCR product of 1.8 kilobases which indicates a probable rearrangement of the p53 coding region either by insertion of foreign sequences or by partial duplication of the introduced p53 cDNA. Southern blot analysis of PCR-positive clones confirmed the presence of exogenous p53 cDNA sequences in all X833 mutant p53 clones analyzed (8 of 8) and in most (7 of 9) wild-type p53 clones (data not shown). However, only a few of the wild-type clones (3 of 9, or 33%) displayed the expected 1.8-kilobase hybridization signal, the other clones being associated with larger-size hybridization signals corresponding to plasmid DNA rearrangement. In contrast, nearly all mutant clones (6 of 7, or 86%) displayed the expected 1.8-kilobase band, which suggests that the cDNA has not been rearranged upon integration. These results not only confirm the PCR analysis carried out on the mutant p53 transfectants but also show a further decrease in the number of clones harboring a normal copy of wild-type p53 cDNA.

Northern blot analysis of these transfection clones was then performed to examine p53 mRNA expression (Fig. 1*B*). All transfection clones analyzed, as well as parental Hut292DM cells, express a 2.8-kilobase band which corresponds to endogenous p53 mRNA. This band appears to be of comparable intensity for all clones analyzed and therefore indicates equal RNA loading of all samples. The exogenous p53 mRNA can be differentiated in this analysis from endogenous mRNA due to its smaller size of 2.65 kilobases. The majority (5 of 8, or 63%) of the mutant p53 clones analyzed expressed very high levels of the 2.65-kilobase exogenous mRNA species. In contrast, no normal-size transcript characteristic of exogenous p53 was detected in any of the wild-type p53 clones analyzed. The same analysis performed on a series of separate transfection clones (experiment X813) was also performed and resulted in similar findings. Briefly, PCR analysis for the presence of p53 cDNA was positive for the majority of mutant X813 transfection clones analyzed (7 of 12, or 58%) whereas only two wild-type X813 clones were positive in this assay (2 of 12, or 17%). Northern blot analysis showed high levels of expression of the exogenous p53 transcript in most of the X813 mutant p53 transfection clones analyzed, whereas the X813 wild-type p53 clones positive by PCR analysis failed to express the exogenous transcript.

Two of the wild-type p53 transfection clones (X833.W2 and W18), however, did express high levels of aberrant p53 mRNA transcripts, a finding which prompted us to analyze whether these transcripts might be translated. Fig. 1*C* shows the expression of p53 protein by clones previously analyzed for p53 mRNA expression. As with the Northern blot analysis, p53 antigen was easily detected in most mutant p53 clones analyzed (7 of 8, or 88%), which is in agreement with the prolonged half-life of mutant forms of p53 compared to that of wild-type. In contrast, a very faint band corresponding to endogenous p53 protein was detected in parental Hut292DM cells as well as in all of the wild-type clones. No increase in the expression level of this M_r 53,000 band was observed in the wild-type p53 clones, which correlates with the absence of normal-size exogenous transcript detected by Northern analysis. Several wild-type clones did, however, express high levels of truncated forms of

Table 1 *Transfection efficiencies of wild-type and mutant p53 expression vectors in Hut292DM cells*

Three separate transfection experiments (X813, X832, X833) were performed using Hut292DM cells and the plasmid DNA constructs pC53SN3 and pC53SCX3, which contain a human p53 cDNA (wild-type or mutated at codon 143, respectively) under transcriptional control of the human cytomegalovirus promoter, as well as pCMVneoBam (vector DNA only). Transfection efficiencies are expressed as the total number of geneticin-resistant colonies obtained using 20 μg plasmid DNA and 2×10^6 cells and after 4 weeks of selection.

Experiment	pC53SN3 (wild-type)	pC53SCX3 (mutant)	pCMVneoBam (vector)
X813	115	600	720
X832	55	240	390
X833	780	1900	2520

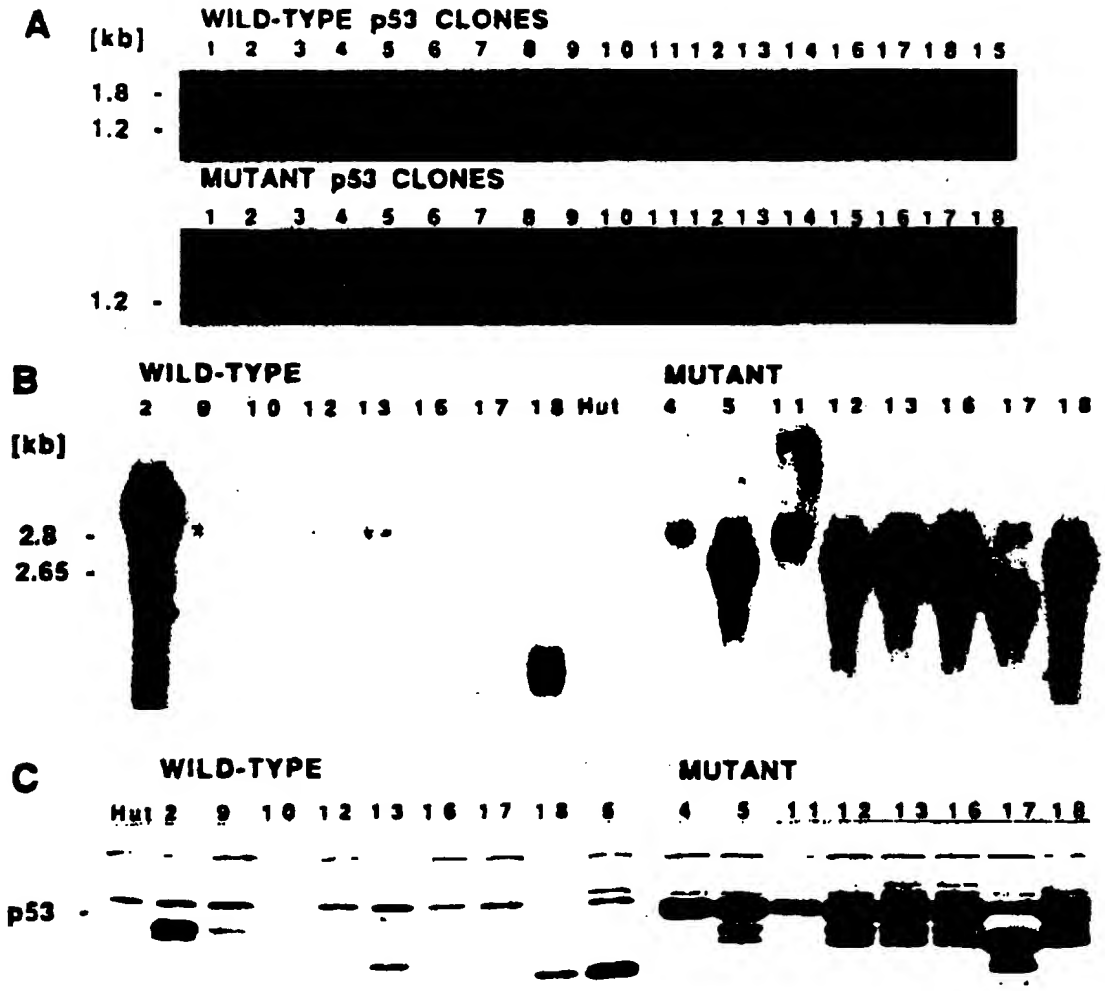

Fig. 1. Analysis of exogenous p53 expression in a series of wild-type and mutant p53 transfection clones derived from Hut292DM cells (transfection experiment: X833). *A*, PCR amplification of a 1.2-kilobase fragment corresponding to the full-length coding region of p53 cDNA. *Top*, wild-type p53 transfection clones; *bottom*, mutant p53 clones. *B*, Northern blot analysis of wild-type (*left*) and mutant p53 (*right*) transfection clones as well as control parental Hut292DM cells (*Lane Hut*). Endogenous p53 mRNA migrates at a position corresponding to 2.8 kilobases and may be differentiated from exogenous p53 mRNA (2.65 kilobases). *C*, Western blot analysis of p53 antigen using the same cells as in *B*. The position of migration of the normal p53 is indicated.

p53 protein. These truncated p53 variants appear to be related to the aberrant mRNA transcripts expressed by these clones. In particular, clones X833.W2 and W18, which were associated with a larger and smaller size mRNA transcript, respectively, both appear to synthesize, respectively, truncated M_r ~45,000 and ~33,000 p53 proteins. Two additional bands of higher molecular weight (approximately 55,000 and 75,000) were detected in all samples analyzed; these probably represent common epitopes shared by proteins unrelated to p53 but recognized by the monoclonal antibody used in this assay.

Selected clones were further analyzed for their growth properties in vitro (Fig. 2). The growth rates (*e.g.*, doubling times) and saturation densities of various mutant p53 clones did not vary significantly from that of parental Hut292DM cells. Furthermore, wild-type p53-transfected cells shown to express high levels of truncated p53 protein exhibited similar *in vitro* growth properties as parental Hut292DM cells or cells transfected with the control vector only (data not shown). The results, however, were different when these transfected clones were assayed *in vivo* for their tumorigenic potential (Fig. 3). A particular wild-type p53 transfection clone (X833.W2) consistently exhibited a reduced tumorigenic potential compared to parental or vector transfected cells. Interestingly, X833.W2 cells were shown by Western blot analysis to express what appears to be a mildly truncated form of the p53 protein with a molecular weight of approximately 45,000. Moreover, all other forms of truncated p53 protein of smaller size did not appear to affect the tumorigenic potential of Hut292DM transfection clones. Various mutant p53 transfection clones, tested under identical experimental conditions, displayed a tumorigenic potential similar to that of parental or vector transfected Hut292DM cells (data not shown).

The results of the molecular analysis of p53 expression in wild-type and mutant p53 transfection clones suggest that growth suppression is mediated by high expression levels of wild-type p53 in this human lung cancer cell line. Indeed, the

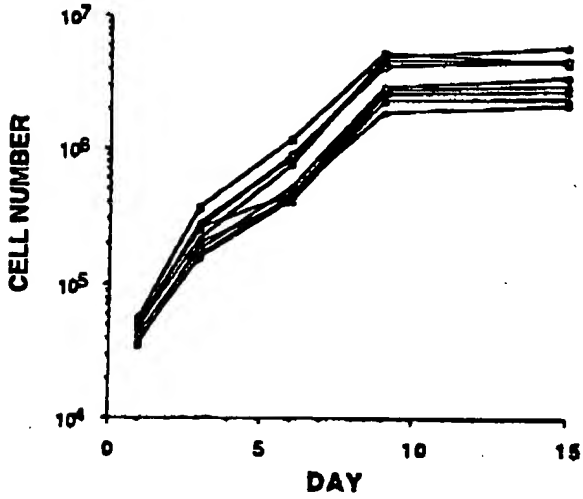

Fig. 2. *In vitro* growth rate analysis of Hut292DM parental cells as well as four mutant p53 and four control-vector-only transfection clones. Cell lines analyzed are: Hut292DM (×); mutant p53 transfection clones, X833.M12 (■), -M13 (●), -M16 (▲), -M18 (◆); vector-only clones, X833.N1 (□), -N2 (○), -N4 (△), -N6 (◇). For details on the experimental procedure followed, see "Materials and Methods."

clonal analysis, repeated twice on two separate transfectionexperiments, consistently failed to identify any wild-type transfection clones expressing a normal exogenous p53 protein, whereas exogenous mutant p53 appeared to be stably expressed at high levels in nearly all clones analyzed. We conclude from these results that expression of exogenous wild-type p53 is incompatible with the sustained proliferation of Hut292DM cells, a finding consistent with the transfection efficiencies obtained with the wild-type and mutant cDNA constructs (see Table 1). These results also demonstrate that a single point mutation at codon 143 (a transition from an alanine to a valine residue) results in the apparent loss of function of p53 with respect to its growth-inhibitory properties for Hut292DM cells, since no difference in transfection efficiency was noted using either the mutant p53 cDNA or the control vector. Several studies involving human colon, breast, and lung cancer cells (2, 4, 5) have also shown that restoration of normal p53 protein expression in otherwise defective cell lines (either null for p53 expression or carrying a p53 mutation) severely affected the growth capacity of these model cell lines. This indicates that the loss of function resulting from p53 alterations may represent a critical step in human carcinogenesis and could lead to some form of gene therapy based on the restoration of expression of p53 in defective cell lines. However, previous efforts to influence the growth potential of tumor cells expressing endogenous wild-type p53 by transfection of the p53 gene were not successful (2, 4), although the reason for this failure is poorly understood. We now demonstrate that expression of exogenous wild-type p53 may lead to the growth arrest of highly proliferative tumor cells constitutively expressing endogenous wild-type p53. The status of the *p53* gene in this cell line was analyzed previously by full-length sequencing of the entire coding region (14). Hut292DM cells do not contain a p53 mutation, consistent with our present Western blot analysis showing the expression of trace amounts of p53 protein in these cells. At present, we may only speculate as to how exogenous expression of p53 results in growth suppression of Hut292DM cells, perhaps by overwhelming the normal regulatory mechanism of p53 function. To date, possible mechanisms include p53 phosphorylation or interaction with another protein (18). An alternative explanation for the capability of additional exogenous wild-type p53 to inhibit cell growth may reside in the very short half-life associated with wild-type p53 protein as compared with the prolonged half-life of mutant forms of the protein. It may be argued that specific proteases are involved in the degradation and elimination of p53 function throughout the cell cycle and that when a critical level of p53 protein synthesis is reached these proteolytic events are no longer able to control overexpression of wild-type p53. Furthermore, a negative feedback mechanism for p53 expression has recently been described whereby p53 represses the transcriptional activity of the p53 natural promoter (19). Evidently, this negative regulatory mechanism was not effective in our transfection studies, which used a cytomegalovirus heterologous promoter for exogenous p53 expression. We do not necessarily favor a mere toxic effect elicited by high levels of wild-type p53 expression, as previous studies using identical expression vectors have shown that certain tumor cells tolerate such levels of expression without any observed effect (2, 4). Therefore, our present work extends the scope of the potential effectiveness of wild-type p53 to control tumor growth to recipient cells that contain no apparent defect in endogenous wild-type p53 expression. As our knowledge of p53 regulatory mechanisms increases it will be of interest to compare our results with those of other model systems, which may help understand why Hut292DM cells are sensitive to exogenous wild-type p53 expression.

Our data also indicate that although expression of exogenous p53 is incompatible with Hut292DM cell growth *in vitro*, we were able to isolate stable transfection clones expressing various truncated forms of p53 protein. Cells expressing high levels of truncated p53 protein were not, however, affected in their *in vitro* growth properties. This may indicate that a critical domain of the molecule which mediates tumor cell growth control *in vitro* has been deleted from the final translation product, possibly as a result of gene rearrangement or point mutation leading to a stop codon. The lack of any apparent inhibition of

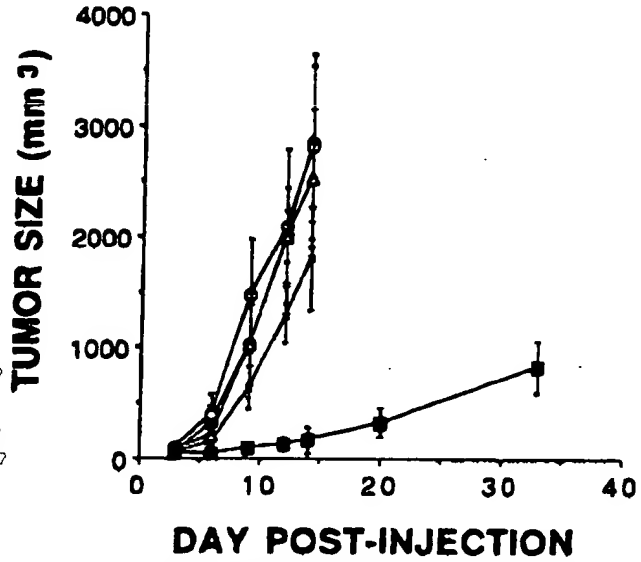

Fig. 3. Tumorigenicity assay in nude mice of Hut292DM parental cells as well as wild-type and mutant p53 transfection clones. Cell lines analyzed are: Hut292DM (×); wild-type p53 transfection clone X833.W2 (■); mutant p53 clones X833.M13 (●), and -M18 (○); and vector-only transfection clone X833.N2 (△). For details on the experimental protocol, see "Materials and Methods."

growth *in vitro* displayed by cells expressing various truncated p53 protein was in sharp contrast to the tumorigenic potential of X833.W2 cells, which synthesize a high level of a M_r 45,000 p53 truncated protein and consistently displayed growth inhibition *in vivo*. Although our observations to date are based on a single clonal event, the results suggest that growth control mechanisms mediated by p53 differ *in vitro* from *in vivo* situations and that separate domains of the p53 molecule may be responsible for these effects. We are now in the process of sequencing these altered forms of p53 in order to obtain a better definition of the domains possibly involved in growth regulation observed *in vitro* and *in vivo*. Although we are only beginning to understand the mechanisms by which the *p53* tumor suppressor gene may control abnormal proliferation of cancer cells, this study provides original evidence that a human lung cancer cell line expressing endogenous wild-type p53 can be growth inhibited by high levels of p53 expression. These results should also provide useful information on the domain-function organization of the p53 protein.

References

1. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. p53 mutations in human cancers. Science (Washington, DC), *253:* 49–53, 1991.
2. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K. V., and Vogelstein, B. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science (Washington DC), *249:* 912–915, 1990.
3. Mercer, W. E., Shields, M. T., Amin, M., Sauve, G. J., Appella, E., Romano, J. R., and Ullrich, S. J. Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. Proc. Natl. Acad. Sci. USA, *87:* 6166–6170, 1990.
4. Casey, G., Lo-Hsueh, M., Lopez, M. E., Vogelstein, B., and Stanbridge, E. J. Growth suppression of human breast cancer cells by introduction of a wild-type *p53* gene. Oncogene, *6:* 1791–1797, 1991.
5. Takahashi, T., Carbone, D., Takahashi, T., Nau, M. N., Hida, T., Linnoila, I., Ueda, R., and Minna, J. D. Wild-type but not mutant *p53* suppresses the growth of human lung cancer cells bearing multiple genetic lesions. Cancer Res., *52:* 2340–2343, 1992.
6. Mercer, W. E., Nelson, D., DeLeo, A. B., Old, L. J., and Baserga, R. Microinjection of monoclonal antibody to protein p53 inhibits serum-induced DNA synthesis in 3T3 cells. Proc. Natl. Acad. Sci. USA, *79:* 6309–6312, 1982.
7. El-Deiry, W., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., and Vogelstein, B. Definition of a consensus binding site for p53. Genetics, *1:* 45–49, 1992.
8. Funk, W. D., Pak, D. T., Karas, R. H., Wright, W. E., and Shay, J. W. A transcriptionally active DNA-binding site for human p53 protein complexes. Mol. Cell. Biol., *12:* 2866–2871, 1992.
9. Fields, S., and Jang, S. K. Presence of a potent transcription activating sequence in the p53 protein. Science (Washington DC), *249:* 1046–1049, 1990.
10. Bischoff, J. R., Friedman, P. N., Marshak, D. R., Prives, C., and Beach, D. Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2. Proc. Natl. Acad. Sci. USA, *87:* 4766–4770, 1990.
11. Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell, *69:* 1237–1245, 1992.
12. Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Milburn Jessup, J., vanTuinen P., Ledbetter, D. H., Barker, D. F., Nakamura, Y., White, R., and Vogelstein, B. Chromosome 17 deletions and *p53* gene mutations in colorectal carcinomas. Science (Washington DC), *244:* 217–221, 1989.
13. Nigro, J. M., Baker, S. J., Preisinger, A. C., Milburn Jessup, J., Hosseter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F. S., Weston, A., Modali, R., Harris, C. C., and Vogelstein, B. Mutations in the *p53* gene occur in diverse human tumour types. Nature (Lond.), *342:* 705–708, 1989.
14. Lehman, T. E., Bennett, W. P., Metcalf, R. A., Welsh, J. A., Ecker, J., Modali, R. V., Ullrich, S., Romano, J. W., Appella, E., Testa, J. R., Gerwin, B. I., and Harris, C. C. p53 mutations, *ras* mutations, and p53-heat shock 70 protein complexes in human lung carcinoma cell lines. Cancer Res., *51:* 4090–4096, 1991.
15. Sambrook, J. E., Fritsch, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual, Ed. 2. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
16. Gough, N. M. Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. Anal. Biochem., *173:* 93–95, 1988.
17. Harlow, E., and Lane, D. Antibodies: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1988.
18. Montenarh, M. Functional implications of the growth-suppressor/oncoprotein p53 (review). Int. J. Oncol., *1:* 37–45, 1992.
19. Ginsberg, D., Mechta, F., Yaniv, M., and Oren, M. Wild-type p53 can down-modulate the activity of various promoters. Proc. Natl. Acad. Sci. USA, *88:* 9979–9983, 1991.

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Cytotoxic Effects of Adenovirus-mediated Wild-Type p53 Protein Expression in Normal and Tumor Mammary Epithelial Cells

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ABSTRACT

To evaluate the effects of the wild-type p53 expression in normal and tumor cells, we have constructed a recombinant adenovirus vector (E1 minus) expressing human wild-type p53 cDNA (AdWTp53). Infection of normal and tumor cells of lung and mammary epithelial origin with AdWTp53 resulted in high levels of wild-type p53 expression. Production of p53 protein following infection was dependent on the dose of AdWTp53 with maximum amounts of p53 produced following infection with 50 plaque-forming units/cell. AdWTp53 infection inhibited the growth of all human cell lines studied. However, tumor cells that were null for p53 prior to infection (H-358 and MDA-MB-157) and tumor cells that expressed mutant endogenous p53 protein (MDA-MB-231 and MDA-MB-453) were more sensitive to AdWTp53 cytotoxicity than cells that contained the wild-type p53 (MCF-7, MCF-10, 184B5, and normal mammary epithelial cells). All cells exhibited WAF1/Cip1 mRNA and protein induction following AdWTp53 infection. AdWTp53-induced cytotoxicity of human tumor cell lines expressing mutant *p53* was mediated by apoptosis as revealed by nucleosomal DNA fragmentation analysis. No detectable nucleosomal DNA fragmentation was observed following AdWTp53 infection of human cells expressing wild-type p53. These data suggest that endogenous p53 status is a determinant of AdWTp53-mediated cell killing of human tumor cells.

INTRODUCTION

The tumor suppressor gene *p53* is apparently the most frequently altered gene analyzed in human tumors, including those from breast and lung (1–3). Recently, there has been an increasing interest in elucidating the mechanisms by which *p53* mediates its functions in normal cells, how various mutations in *p53* are responsible for aberrant cell growth (1, 4), and the possibility of using wild-type *p53* in gene therapy (5). It is therefore important to understand the biological consequences of the wild-type *p53* overexpression in both normal and tumor cells. Different approaches have been used to study the effects of p53 expression in cells, including exposure of cells to UV radiation and DNA damaging agents (6–9), both of which have been shown to induce increased expression of cellular p53. Alternative genetic approaches have also been used, including introduction of a temperature-sensitive mutant of *p53* (10–12) or gene knock-out experiments (13) to alter intracellular p53 expression and function. There is compelling evidence that wild-type p53 can negatively influence cell growth by causing G_1 arrest (8, 10) and/or by inducing apoptosis (6, 7, 11, 12, 14, 15). In cells expressing mutant *p53*, these effects of wild-type p53 are abrogated, resulting in abnormal cell growth (1, 4, 15).

One approach to understanding the role of p53 protein is to utilize p53 expression vectors capable of producing high levels of p53 protein in cells. This strategy not only allows the study of the role of p53 in the control of regulation of cell growth in both normal and malignant cells, but also has implications in gene therapy for cancers which are null for *p53* or express mutated *p53*. Although plasmids and retroviruses have been used to express p53 protein (4, 14, 16), the efficiency of these transfection techniques is generally low. To study the effects of wild-type p53 expression in normal and transformed epithelial cells, we have generated a replication-deficient Ad[3] containing a human wild-type p53 cDNA (AdWTp53). The choice of adenovirus vector was made because adenovirus-based vectors can grow to high titers (17, 18), are internalized into cells with an efficient receptor-mediated endocytosis (19, 20), are replication incompetent (21, 22), and express a transgene to high levels in epithelial cells (20, 22). We have used this vector to express wild-type p53 protein in tumor and normal cells with different intrinsic p53 status (null, mutant, or wild type) to (*a*) examine the effects of high levels of p53 on the growth properties of normal and tumor cells, (*b*) examine the effects of p53 expression on the induction of *WAF1/Cip1* and *mdm2* gene expression, and (*c*) evaluate the role of apoptosis in p53-mediated cytotoxicity. These studies indicate that an adenovirus vector expressing wild-type p53 is markedly cytotoxic to tumor cells that have null or mutant *p53* expression, and that this vector can provide a useful tool to study the precise molecular mechanisms by which p53 mediates its effects in normal and transformed cells.

MATERIALS AND METHODS

Construction of an AdWTp53. AdWTp53 was constructed using cotransfection of shuttle vector pDK10 containing the wild-type p53 expression cassette and a plasmid pJM17 containing the adenovirus type 5 genome. The shuttle vector

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[3] The abbreviations used are: Ad, adenovirus; FBS, fetal bovine serum; EGF, epidermal growth factor; NMEC, normal mammary epithelial cell; pfu, plaque-forming unit; Adr^R, Adriamycin resistant; IC_{50}, 50% inhibitory concentration; β-gal, β-galactosidase.

pDK10 was constructed by inserting the human cytomegalovirus immediate early promotor and enhancer, a 1.7-kb *Xba*I fragment of human p53 cDNA (23), the SV40 small T intron, and SV40 polyadenylation signal into the *Cla*I site of plasmid pXCX2 (18). Plasmid pDK10 was cotransfected with pJM17 (Ref. 24; kindly provided by F. Graham, McMaster University, Hamilton, Ontario, Canada) into the transformed human embryonic kidney cell line 293 (ATCC CRL1573) by calcium phosphate-mediated gene transfer technique (Refs. 18 and 25; GIBCO-BRL, Gaithersburg, MD). The day following transfection, the medium was replaced with a 1X MEM (GIBCO-BRL) containing 1% sea plaque agarose gel (FMC, Rockland, ME) and 10% FBS (GIBCO-BRL), and the cells were incubated at 37°C. Every five days 2 ml MEM containing 1% sea plaque agarose gel and 10% FBS were added to the top of the cells until plaques were observed. Isolated plaques were picked and subjected to another cycle of infection in 293 cells as described previously (18, 25).

Purified recombinant AdWTp53 viruses were assayed for the absence of E1a and the presence of p53 sequences using PCRs (26). Cell lysates were prepared 24 h following infection with adenoviral vector using guanidine thiocyanate solution, and aliquots were used for PCR analysis. For E1a analysis, the primers used were 5'-TCTTGAGTGCCAGCGAGTAG-3' and 5'-CAAGGTTTGGCATAGAAACC-3'. For p53, one primer (5'-GTTGGCTCTGACTGTACC-3') was selected from exon 7, and the downstream primer 5'-GTTCCGTCCCAGTAGATTACC-3' was selected from the exon 8. This combination of primers can differentiate the PCR product of the endogenous genomic *p53* gene from the viral-associated *p53* gene. AdWTp53 was propagated in 293 cells grown in monolayers, purified by two cesium chloride density gradients, dialyzed against a buffer containing 10% glycerol, 1 mM $MgCl_2$ (pH 7.5), and stored at −70°C as described previously (20).

Control adenovirus vectors used in this study were: Ad.RSVβgal, an adenovirus vector containing β-galactosidase gene (27), and AdControl, an adenovirus vector dl312 containing no insert (Ref. 21, kindly provided by T. Shenk, Princeton, NJ).

Cell Culture. Breast cancer cell lines [MDA-MB-231 (ATCC HTB26), MCF-7 (kindly provided by R. Buick, University of Toronto, Toronto, Ontario, Canada), and Adr^R MCF-7 (28)] were cultured in α-MEM (GIBCO-BRL) supplemented with 10 mM HEPES, 2 mM glutamine, 0.1 mM nonessential amino acids, 10% FBS, 1 ng/ml EGF, and 2 μg/ml insulin (29). H-358, a lung cancer cell line (kindly provided by J. Minna, University of Texas, Dallas, TX), and MDA-MB-453 cells, a breast cancer cell line (ATCC HTB131), were grown in RPMI 1640 containing 10% FBS. MDA-MB-157 (ATCC HTB 24), a breast cancer cell line, was grown in improved minimum essential medium (IMEM) (GIBCO-BRL) supplemented with 10% FBS and 0.5% Redu-Ser II (Upstate Biotechnology Inc., Lake Placid, NY). NMECs derived from reduction mammoplasties (CC-201 6; Clonetics Corp., San Diego, CA), and 184B5 cells, immortalized mammary epithelial cells (ATCC CRL10317), were cultured in mammary epithelial basal medium (Clonetics Corp.) supplemented with 1X vitamins, 0.5% FBS, 20 ng/ml EGF, 5 μg/ml hydrocortisone, and 52 μg/ml bovine pituitary extract (29). Immortalized MCF10 cells (kindly provided by S. Brooks, Michigan Cancer Foundation, Detroit, MI) were cultured in DMEM/F12 (GIBCO-BRL) supplemented with 2.5% horse serum (GIBCO-BRL), 10 mM HEPES (Calbiochem, La Jolla, CA), 2 mM glutamine (Biofluids, Rockville, MD), 0.1 mM nonessential amino acids (GIBCO-BRL), 20 ng/ml EGF (Upstate Biotechnology), 10 μg/ml insulin (Bochringer Mannheim, Indianapolis, IN), and 0.5 μg/ml hydrocortisone. 293 (ATCC CRL 1573), an adenovirus-transformed human embryonic kidney cell line, was cultured in improved MEM (Biofluids) supplemented with 2 mM glutamine (Biofluids), 2.5 μg/ml Fungizone (Biofluids), 100 units/ml penicillin, 100 μg/ml streptomycin (100×Pen-Strep; Biofluids), and 10% FBS.

Effect of AdWTp53 on Cell Growth. To study the effect of adenovirus vectors on cell growth, 5×10^4 cells were plated in each well of 6-well dishes. After 24 h, cells were exposed to AdWTp53 or AdControl (10 pfu/cell) in medium containing 2% FBS. After an incubation of 2 h at 37°C, serum concentration in the medium was raised to 10% and incubations continued at 37°C. Cells were trypsinized on each day and counted using a hematocytometer. Cytotoxicity of adenovirus vectors was assessed using a colorimetric assay as described previously (30). Briefly, 500 cells were plated in each well of 96-well plates and incubated for 24 h. Cells were then exposed to the appropriate cell growth medium except that the concentration of the serum (if it was a component of the growth medium) was reduced to 2%. Different doses of adenovirus vectors were included in the incubation medium (several 5-fold dilutions). After 2-h incubation at 37°C, the serum concentration was increased to 10%, and the cells were incubated for 7 days at 37°C. Cells were fixed by the addition of ice-cold 50% trichloroacetic acid (added onto the top of the medium in each well to a final concentration of 10%), incubated at 4°C for 1 h, washed five times with water, and then air dried. Trichloroacetic acid-fixed cells were stained for 20 min with 0.4% (w/v) sulforhodamine B (Sigma, St. Louis, MO) dissolved in 1% acetic acid followed by rinsing four times with 1% acetic acid. An A_{564} was obtained using a Bio Kinetics Reader EL340 (Bio-Tek Instruments) and was used as a measure of cell number. The percentage of survival rates of cells exposed to adenovirus vectors were calculated by assuming the survival rate of uninfected cells to be 100%.

β-Galactosidase Activity following Ad.RSVβ-gal Infection. The expression of an adenovirus vector containing the β-galactosidase gene was examined by plating 2×10^4 cells in each well of a 96-well plate. The cells were exposed 24 h later to various concentrations of Ad.RSVβ-gal (0.1–500 pfu/cell) in medium used by each respective cell line except that the serum concentration (if required) was reduced to 2%. After 2-h incubation at 37°C, the serum concentration (if required) was raised to 10%, and then cells were incubated at 37°C for an additional 24 h. Cells were then washed three times with PBS (pH 7.5) and lysed in 50 μl 0.1 M Tris (pH 7.5) containing 0.1% Triton X-100. An aliquot (30 μl) was assayed for β-galactosidase activity using a modified protocol (27). Samples were transferred to each well of 96- well plates and treated with 100 μl 20 mM Tris (pH 7.5) containing 1 mM $MgCl_2$, 450 μM β-mercaptoethanol, and 150 μM *O*-nitrophenyl-β-galactopyranoside. Incubations were performed at 37°C for 20 min, and the reaction was stopped by the addition of 150 μl/well of 1 M Na_2CO_3. The

absorbance was determined at 420 nm. An A_{420} of 1 was defined as 1 unit of enzyme activity.

Immunoprecipitation of p53 Protein in Cells Infected with AdWTp53. Cells (1×10^6) were plated in 10-cm dishes and infected with AdWTp53 or AdControl for 24 h as described above. Immunoprecipitations were performed using an anti-p53 antibody essentially as described (31). In brief, cells were incubated with 3 ml methionine-free DMEM (Biofluids) containing 5% dialyzed FCS (Biofluids) and 100 μCi/ml [^{35}S]methionine and [^{35}S]cysteine mixture (Expre ^{35}S^{35}S-protein labeling mix, 1000 Ci/mmol; New England Nuclear) for 2 h. Cells were washed with ice-cold PBS and solubilized at 4°C in buffer A [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% NP40, 0.1% sodium deoxycholate (Sigma), 0.5% sodium lauryl sulfate (SDS) (Research Genetics, Huntsville, AL), 1 mM phenylmethyl sulfonylfluoride, 10 μg/ml aprotinin, 1.0 μg/ml leupeptin, and 1.0 μg/ml pepstatin (all protease inhibitors from Boehringer Mannheim)]. Aliquots of 500 μl ^{35}S-labeled lysates were incubated with a 1:50 dilution of anti-p53 mAb PAb 1801 (Ab-2; Oncogene Science, Uniondale, NY) at 4°C for 1 h, after which 15 μl protein A/G agarose (Oncogene Science) were added, and the incubations were continued for an additional 1 h with rotation. Samples were then centrifuged at 5000 × *g* for 5 min, and the pellets were washed successively with buffer A, buffer A containing 1 M NaCl, and finally with buffer A again. SDS gel sample buffer (50 μl) was added, and the samples were heated for 5 min at 95°C to elute proteins from the immunoadsorbent. The tubes were centrifuged again at 5000 × *g* for 5 min, and 20-μl aliquots of protein samples were subjected to SDS-PAGE. Gels were then dried and exposed to X-ray film as described previously (31).

Western Blot Analysis of p53, WAF1/Cip1, and mdm2 Proteins in Cells Infected with AdWTp53. Cells (0.5×10^6) were plated in 6-cm dishes and incubated with AdWTp53 or AdControl for 24 h as described above. Cells were then washed three times with ice-cold PBS, scraped and resuspended in 1 ml 1X SDS-PAGE buffer (62 mM Tris, pH 6.8, 2 mM EDTA, 15% sucrose, 10% glycerol, 3% SDS, and 0.7 M 2-mercaptoethanol), and boiled for 10 min. Equal amounts (15 or 50 μg) of denatured protein were electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose filters (29). Filters were blocked with Tris-buffered saline containing 5% dried milk and 0.1% Tween 20 (Sigma). Blots were probed with 4 μg/ml Ab-2 and Ab-6 for p53, 4 μg/ml EA 10 for WAF1/Cip1, 3 μg/ml IF2 for mdm2, or 3 μg/ml actin (Ab-1) antibody. All antibodies were obtained from Oncogene Science. After incubation with the primary antibodies, the blots were washed with Tris-buffered saline containing 0.1% Tween 20, incubated with horse radish peroxidase conjugated to secondary antibody, and specific complexes were detected by the enhanced chemiluminescence technique according to manufacturer's directions (New England Nuclear).

Northern Blot Analysis of WAF1/Cip1 and mdm2 in Cells Infected with AdWTp53. Cells (2×10^6) were plated in 15-cm dishes and incubated with 10 pfu/cell of adenoviral vectors. After incubation for 24 h at 37°C, RNA was extracted by rinsing cells three times with cold PBS and dissolving the cells in a 2-ml solution of guanidine isothiocyanate. RNA was purified by centrifugation over a 5.7 M cesium chloride cushion (29), fractionated by electrophoresis in agarose gels containing formaldehyde, transferred to Magna NT filters, and cross-linked as described previously (29). Following prehybridization, filters were hybridized using a 2.1-kb fragment of WAF1/Cip1 or an 800-bp fragment from 36B4. Following hybridization, the filters were washed and exposed to X-ray films, and autoradiographs developed as described previously (29).

Detection of Nucleosomal DNA Fragmentation in Cells Infected with AdWTp53. For DNA fragmentation studies, 2×10^6 cells were plated in 10-cm dishes and incubated with adenovirus vectors (50 pfu/cell) for 24 h. Both adherent and floating cells were collected together and pelleted by centrifugation at 1800 × *g* for 5 min (RT-6000B; DuPont, Boston, MA). Cell pellets were rinsed with cold PBS, and low molecular weight DNA was prepared using a modified Hirt extraction method as described previously (22). Briefly, pellets were lysed in 1 ml 10 mM Tris, 10 mM EDTA disodium (pH 7.4; Research Genetics), 0.6% SDS (Research Genetics), and 0.2 mg/ml proteinase K (Boehringer Mannheim). Samples were incubated at 55°C for 5 h, and low molecular weight DNA was prepared using the Hirt extraction method (22) and evaluated by electrophoresis on agarose gel (2%).

The presence of apoptotic cells was also followed by an *in situ* apoptosis detection kit (catalogue number S7110-kit; Oncor, Gaithersburg, MD) according to the manufacturer's instructions.

RESULTS

Construction of an AdWTp53. Homologous recombination between shuttle vector pDK10, containing an expression cassette of human wild-type p53 cDNA and the adenovirus genome cloned in plasmid pJM17, generated an adenovirus clone in which the adenovirus E1 region was replaced by the wild-type p53 cDNA expression cassette. PCR analysis of the purified recombinant adenovirus indicated that it contained p53 cDNA but was devoid of E1a sequences. Fig. 1 is a schematic diagram of AdWTp53. The 5′ end of the genome contains the AdWTp53 expression cassette (10.3 mu) followed by the rest of the adenovirus genome. The key elements of the expression cassette of AdWTp53 include the left inverted terminal repeat, adenoviral origin of replication, encapsidation signal, E1a enhancer, cytomegalovirus immediate early promotor, the human wild-type p53 cDNA, and SV40 polyadenylation signal.

AdWTp53-mediated Synthesis of p53 Protein. To determine whether AdWTp53 expresses the p53 protein in tumor cells, a lung tumor cell line H-358, which lacks endogenous p53 (2), was exposed to various concentrations of either AdControl or AdWTp53 for 24 h. Following infection, immunoprecipitation of p53 was performed as described in "Materials and Methods." As shown in Fig. 2*A*, there was no detectable p53 in H358 cells infected with AdControl. In contrast, p53 protein was easily detected by immunoprecipitation in cells infected with 1 pfu/cell of AdWTp53. Furthermore, the amount of immunoprecipitable p53 protein increased with increasing concentrations of AdWTp53 vector.

To investigate adenovirus-mediated p53 expression in breast tumor cells, we exposed several different mammary cell lines (MCF-7, MCF-10, Adr^R MCF-7, and MDA-MB-231) to AdWTp53 and assessed the synthesis of p53 protein by immu-

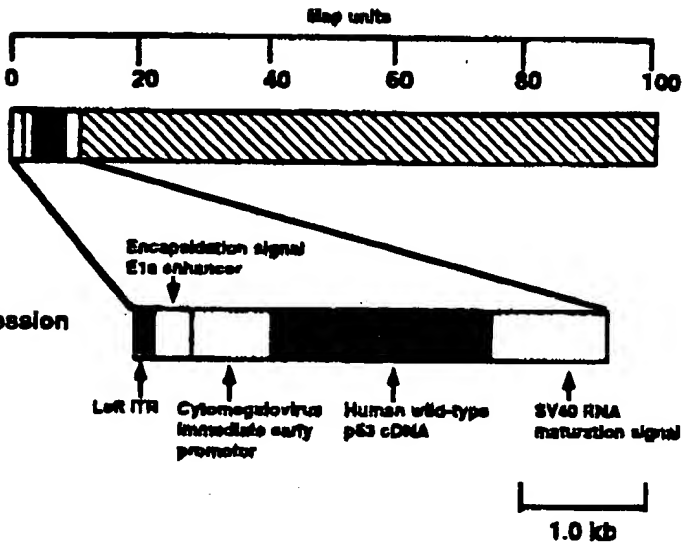

Fig. 1 Structure of the recombinant adenoviral vector AdWTp53. ■, adenovirus type 5 genome of 9.24–100 mu. *Bottom*, enlargement of human wild-type p53 expression cassette. Human wild-type p53 expression cassette contains left inverted terminal repeat (*ITR*), origin of replication, encapsidation signals, and E1a enhancer derived from adenovirus type 5 (*stippled segments*); human cytomegalovirus immediate early promotor (*left blank segment*); human wild-type p53 cDNA (*solid segment*); and SV40 RNA maturation signal (*right blank segment*).

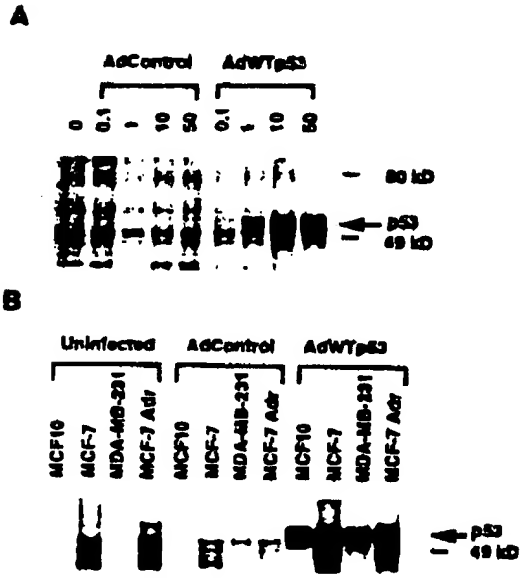

Fig. 2 *A*, immunoprecipitation of ^{35}S-labeled human wild-type p53 protein from H-358 cells exposed to various doses of AdWTp53 or AdControl. H-358 cells were exposed to various concentrations of either AdControl or AdWTp53 for 24 h as described in "Materials and Methods." After labeling the cells with [^{35}S]methionine-cysteine, cell lysates were immunoprecipitated using anti-p53 antibody, solubilized protein samples were loaded on 8% SDS-PAGE, and gels were dried and exposed to X-ray film. *Left panel*, signals of p53 precipitates from H-358 cells exposed to AdControl at 0.1, 1, 10, and 50 pfu/cell and uninfected cells. *Right panel*, signals of p53 precipitates from H-358 cells exposed to AdWTp53 at 0.1, 1, 10, and 50 pfu/cell. *Numbers 0–50 on top of the lanes*, pfu/cell. *Arrow*, position of p53 protein on gels. *B*, immunoprecipitation of ^{35}S-labeled p53 protein from various cell lines. Various cells (MCF-10, MCF-7, MDA-MB-231, and Adr^R MCF-7) were exposed to AdWTp53 (50 pfu/cell) or AdControl (50 pfu/cell), and p53 protein was immunoprecipitated as described above. *Left panel*, results of p53 immunoprecipitation of uninfected cells; *middle panel*, immunoprecipitation of cells exposed to AdControl; and *right panel*, results of cells exposed to AdWTp53. *Arrow*, position of p53 protein on gels.

noprecipitation. As shown in Fig. 2*B*, MCF-10, MCF-7, Adr^R MCF-7, and MDA-MB-231 expressed low levels of endogenous p53. However, following exposure of the cells to 10 pfu/cell of AdWTp53, a marked increase in the level of p53 protein was observed in the infected cells. In contrast, infection with AdControl did not result in any increase in p53 expression above that present in uninfected cells. These results were also confirmed by Western blot analysis (see below) and indicate that AdWTp53 can infect both human mammary and lung cells. Moreover, infection with AdWTp53 resulted in high levels of p53 expression in these cells.

Effect of AdWTp53 on Cell Growth. We investigated the effect of high-level wild-type p53 expression on the growth of cells having different p53 status. For these studies we used H-358 lung cancer cells, which are devoid of p53 (p53 null; Ref. 2), MDA-MB-231 human breast cancer cells, which express mutant p53 (32), and MCF-7 human breast cancer cells, which express wild-type p53 (16, 32). Each cell line was exposed to 10 pfu/cell of either AdWTp53 or AdControl and harvested daily for cell counts. As shown in Fig. 3, *A* and *B*, infection of H-358 and MDA-MB-231 cells with AdWTp53 completely inhibited cell growth over the 4-day period examined. In both of these cell lines, the cell number was reduced by day 4 to levels less than one-half of that present at time 0. In contrast, MCF-7 cells continued to proliferate although at a slower rate than control cells (Fig. 3*C*). As a control for these experiments, we show that AdControl virus had very little effect on the growth of these cells (Fig. 3).

These results suggested that infection by AdWTp53 had a more profound growth inhibitory effect on cells that were either deficient in *p53* or expressed a mutant *p53* than on cells that expressed wild-type *p53*. To confirm these observations, we investigated the effects of AdWTp53 on cancer cells that lacked *p53* expression (H-358 and MDA-MB-157; Refs. 2 and 32), cancer cells that expressed endogenous mutant *p53* (MDA-MB-231 and MDA-MB-453; Refs. 32 and 33), cancer cells that

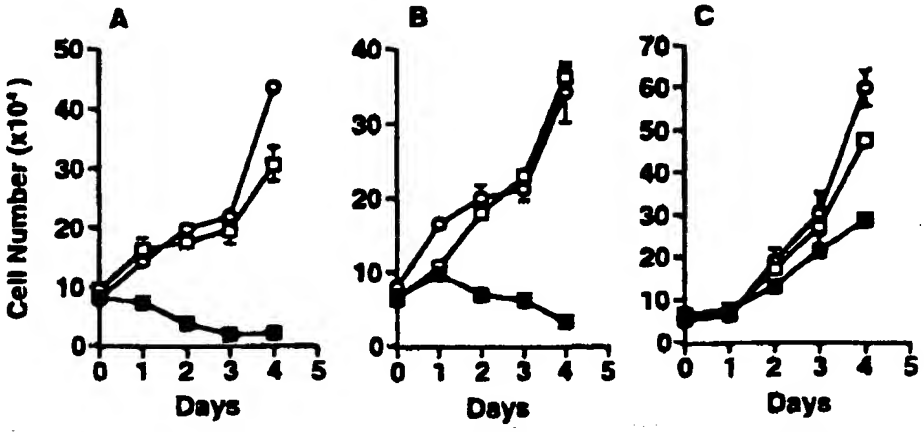

Fig. 3 Effect of AdWTp53 and AdControl on cell growth. Cells (5×10^4) were plated in triplicate on 6-well plates, exposed to AdWTp53 (10 pfu/cells) or AdControl (10 pfu/cell), and cell number counted on each day. Shown are cell number of: (*A*) H-358 cells: uninfected (○), exposed to AdWTp53 (■), and exposed to AdControl (□); (*B*) MDA-MB-231 cells: uninfected (○), exposed to AdWTp53 (■), and exposed to AdControl (□); (*C*) MCF-7 cells: uninfected (○), exposed to AdWTp53 (■), and exposed to AdControl (□). Values shown are mean ± SE.

Table 1 Summary of the endogenous p53 status of the various cell lines, IC_{50} values of AdWTp53 in each cell line, β-gal activity in these cells after infecting with Ad.RSVβgal vector, and relative induction of WAF1/Cip1 protein expression following infection with AdWTp53

Cell line	Endogenous p53 status[a]	IC_{50} AdWTp53[b]	β-gal Activity[c]	Fold induction WAF1/Cip1[d]
H-358	Null	0.17	0.75	105
MDA-MB-157	Null	0.30	0.54	83
MDA-MB-231	Mutant	0.4	0.66	154
MDA-MB-453	Mutant	0.70	0.58	71
MCF-7	Wild type	30	0.30	2.3
184B5	Wild type	4.5	ND	2.4
MCF-10	Wild type	5.5	ND	7.5
NMECs	Wild type	100	0.731	1.2

[a] The status of endogenous p53 in each cell line is from Refs. 2, 16, 29, 32, and 33, and J. Gudas *et al.* unpublished data as described in the text.

[b] Values are estimated from the cell killing experiments described in the text.

[c] β-galactosidase activity in each cell line was measured after infecting cells with 20 pfu/cell as described in "Materials and Methods."

[d] Fold increase in the WAF1/Cip1 expression represents the AdWTp53-mediated (10 pfu/cell) increase in WAF1/Cip1 protein expression over the uninfected cells.

expressed wild-type p53 (MCF-7) and immortalized, and normal mammary epithelial cells that expressed wild-type *p53* (MCF-10, 184B5, and NMECs)[4] (29). In these experiments each cell line was exposed to increasing concentrations of AdWTp53 for 7 days. As shown in Table 1, cells that are null for expression of p53 were the most sensitive to the inhibitory effect of AdWTp53 (IC_{50}s for H-358 and MDA-MB-157 cells were 0.17 and 0.3 pfu/cell, respectively). Cells that express a mutant p53 protein were only slightly less sensitive to the growth inhibitory effects of AdWTp53 (IC_{50}s for MDA-MB-231 and MDA-MB-453 were 0.4 and 0.7 pfu/cell, respectively). In contrast, immortalized or normal cells that expressed wild-type p53 were the most resistant to the cytotoxic effects of AdWTp53, with NMECs being the most resistant (IC_{50}s for 184B5, MCF-10, MCF-7, and NMEC were 4.5, 5.5, 30, and 100 pfu/cell, respectively; Table 1). Although some of the cytotoxic effects of AdWTp53, particularly at high multiplicity of infection (>100 pfu/cell), could be due to the nonspecific effects of the recombinant adenovirus, in general, it appears that cells that express wild-type p53 were 5–250 times more resistant to the AdWTp53-mediated inhibitory effect on cell growth when compared with cells expressing no *p53* or mutant *p53*.

Ad.RSVβ-gal-mediated β-Galactosidase Activity. Since differences in the sensitivity of various cell lines to AdWTp53 could result from either reduced uptake and/or decreased transgene expression, the expression of an adenovirus vector containing the marker gene β-galactosidase was examined in these cells. Cells were exposed to different concentrations of Ad.RSVβ-gal for 24 h, and β-galactosidase activity was measured as described in "Materials and Methods." In all cell lines, expression of β-galactosidase was linear in the range of 1–100 pfu/cell of Ad.RSVβ-gal (data not shown). Following infection of each cell line at 20 pfu/cell, the enzyme activity in each cell line was in the range of 0.3–0.75 units (Table 1). Moreover, as shown below (Fig. 4), each of the cell lines expressed high amounts of p53 when these cells were exposed to AdWTp53. Therefore, the differences in the sensitivity of killing effects of AdWTp53 cannot be explained by alteration in viral uptake and/or differential expression of the transgene.

Effect of AdWTp53 on WAF1/Cip1 and mdm2 Protein Expression. To further investigate the molecular mechanisms underlying the cytotoxicity of AdWTp53, we examined the expression of two cellular proteins that could play a role in mediating the inhibitory effects of p53. These included *WAF1/Cip1*, a gene which is induced in cells and inhibits cyclin kinase

[4] J. Gudas *et al.*, unpublished data.

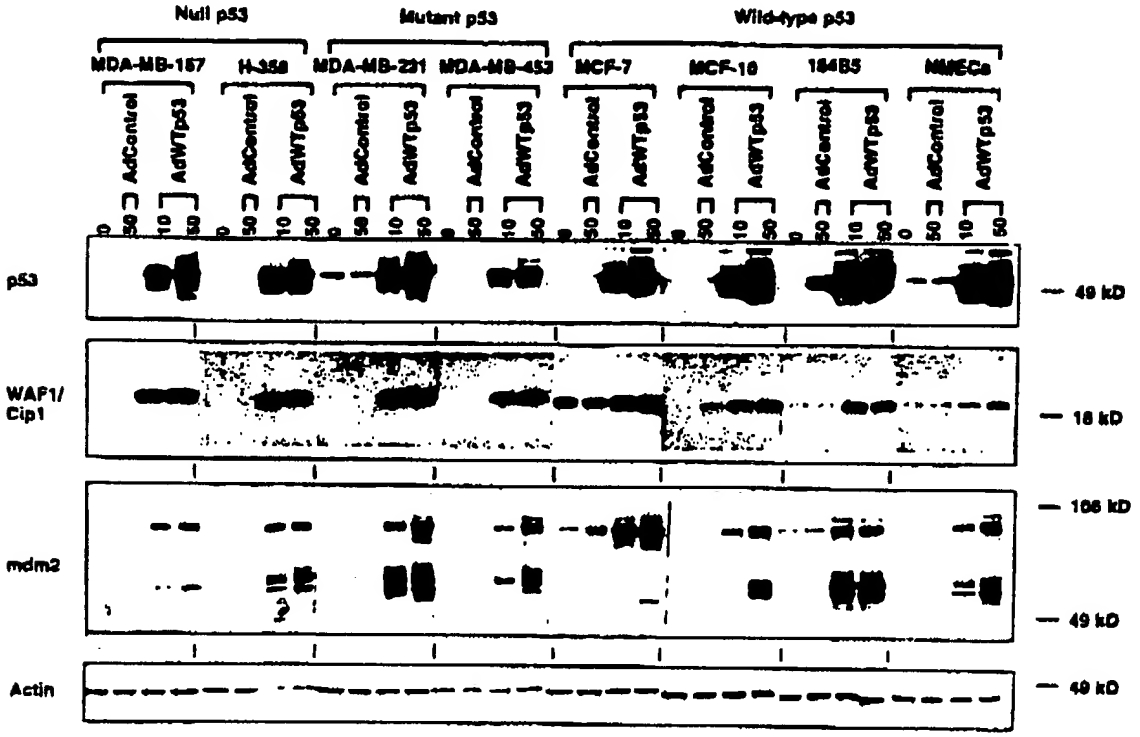

Fig. 4 Western blot analysis of p53, WAF1/Cip1, mdm2, and actin proteins in breast cancer cell lines (MDA-MB-157, MDA-MB-231, MDA-MB-453, and MCF-7), a lung cancer cell line (H-358), immortalized mammary cells (MCF10 and 184B5), and NMECs. Cells (0.5×10^6) were plated in 6-cm dishes and infected with either 10 or 50 pfu/cell of AdWTp53 or 50 pfu/cell of AdControl for 24 h. Cells were harvested and resuspended in 1 ml 1X SDS-PAGE buffer, and 15 μg protein were separated in an 8% SDS-polyacrylamide gel and electroblotted onto nitrocellulose, and the membranes reacted with antibodies corresponding to p53, WAF1/Cip1, mdm2, and actin. Protein bands were detected by autoradiography of X-ray film. *Top of the panel*, type of each cell line used. *Numbers 10 or 50 on top of the lanes*, amount of AdControl or AdWTp53 (pfu/cell). *Left side of the panel*, antibodies used for detecting proteins. *Right side of the panel*, protein molecular weight markers.

(34–36), and *mdm2*, another *p53*-inducible gene that can bind p53 and modulate its function (37).

As shown in Fig. 4, Western blot analyses demonstrated that low levels of endogenous p53 could be detected in all cell lines examined except MDA-MB-157 and H-358. However, the level of p53 increased substantially (at least 10-fold) in each cell line following AdWTp53 infection (10 or 50 pfu/cell). In contrast, the amount of p53 increased little, if at all, above the endogenous p53 protein level in cells exposed to 50 pfu/cell of AdControl. Because cells exposed to either AdControl or AdWTp53 expressed similar levels of actin protein (Fig. 4), increased levels of p53 following AdWTp53 infection cannot be due to loading of different amounts of proteins or other nonspecific mechanisms.

We also examined the induction of WAF1/Cip1 expression following AdWTp53 infection. As shown in Fig. 4, there was little or no detectable basal level of WAF1/Cip1 in cells that did not express endogenous wild-type p53 (MDA-MB-157 and H-358), or in cells that expressed a mutant p53 (MDA-MB-231 and MDA-MB-453), basal levels of WAF1/Cip1 were readily detected in cells that expressed endogenous wild-type p53 (MCF-7, MCF-10, 184B5, and NMECs). Although exposure of cells to 50 pfu/cell of AdControl did not affect the basal level of WAF1/Cip1 in any of these cells, exposure to 10 or 50 pfu/cell of AdWTp53 resulted in a marked increase in WAF1/Cip1 expression in all of the cell line. However, cells expressing endogenous mutant p53 or null for p53 appeared to induce higher levels (fold-induction) of WAF1/Cip1 protein as compared to cells expressing wild-type p53 (Fig. 4 and Table 1).

mdm2 protein levels were also determined before and after AdWTp53 infection in each cell line. Basal levels of mdm2 protein were not detectable in cells that were null for p53 or contained mutant p53. Endogenous mdm2 protein bands of approximately 90 kDa and 57 kDa[4] (37) were readily detected in all cells expressing wild-type p53, and no difference in the levels of either mdm2 proteins were observed following infection of cells with the AdControl vector. In contrast, following exposure to AdWTp53 at 10 or 50 pfu/cell, there was a marked increase in the levels of high and low molecular weight mdm2 proteins in all cell lines examined except MCF-7 cells in which AdWTp53-mediated expression of the 57-KDa protein was minimal.

Effect of AdWTp53 on WAF1/Cip1 mRNA Expression. Since p53 is a DNA-binding transcription factor (38), we determined whether AdWTp53-mediated induction of WAF1/Cip1 protein was regulated at the level of RNA. The expression of WAF1/Cip1 mRNA was assesed by Northern blot analysis following infection of cells with either AdControl or AdWTp53.

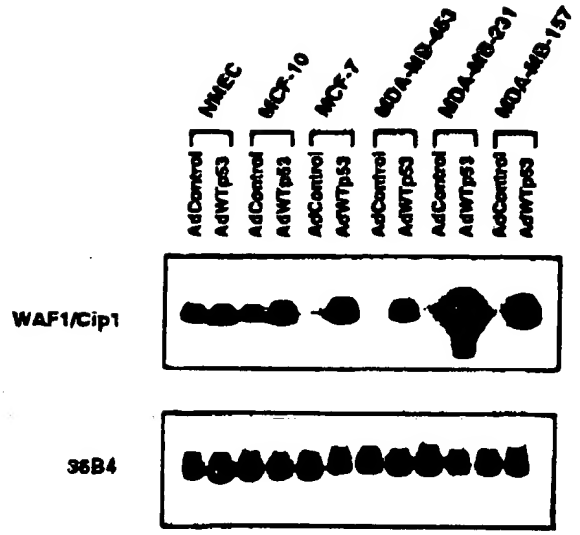

Fig. 5 Northern blot analysis of p53 mRNA in cells exposed to AdWTp53. Cells (2×10^6) were plated and 24 h later infected with AdWTp53 or AdControl (10 pfu/cell) for 24 h, and RNA was prepared and subjected to Northern blot analysis. After transferring RNA to Magna NT membranes, blots were either probed with a p53 or 36B4 cDNA probe. Results of autoradiograms obtained from different cells shown on *top of the lanes*, exposed to either AdControl or AdWTp53.

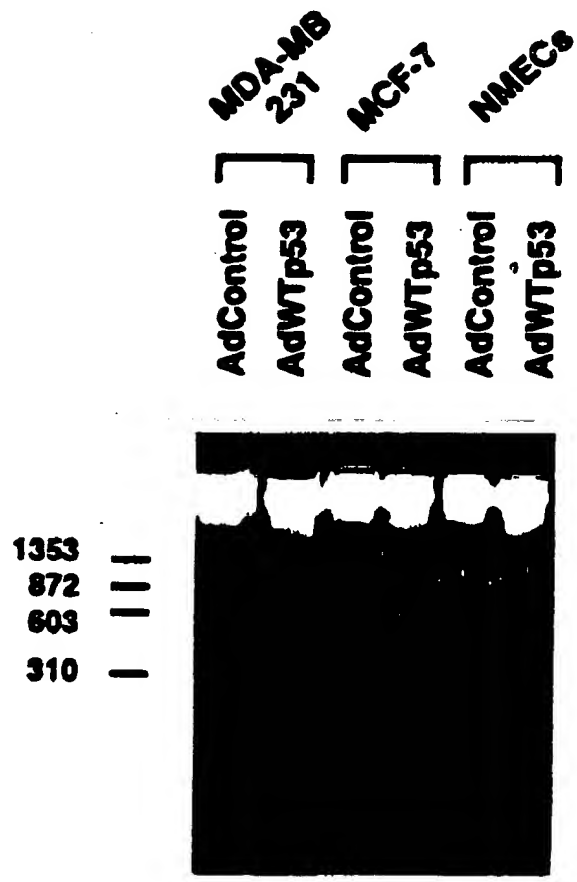

Fig. 6 Nucleosomal DNA fragmentation in AdWTp53-infected MDA-MB-231, MCF-7, and NMECs. Cells (2×10^6) were plated in 10-cm dishes and exposed to either AdControl or AdWTp53. One day after infection, cells were collected and incubated with a lysis buffer, and low molecular weight DNA was prepared and subjected to an agarose gel electrophoresis. Results shown are the DNA pattern observed in various cell lines (*top of the lane*) infected with 50 pfu/cell of either AdControl or AdWTp53. *Numbers on the left*, position of molecular weight markers (bp).

As shown in Fig. 5, cells devoid of WTp53 (MDA-MB-157) and cells expressing mutant *p53* (MDA-MB-453 and MDA-MB-231) had very low levels of WAF1/Cip1 mRNA after infection with AdControl. NMECs, MCF-10 cells, and MCF-7 cells all contained endogenous wild-type p53 and expressed varying levels of WAF1/Cip1 mRNA expression following infection with AdControl. Following infection of AdWTp53, the WAF1/Cip1 mRNA levels in all cell lines increased. There was a 7.4-fold increase in MDA-MB-453, a 21-fold increase in MDA-MB-231, an 8.2-fold increase in MDA-MB-157 cells, a 6-fold increase in MCF-7 cells, a 2-fold increase in MCF-10 cells, and a 1.2-fold increase in NMECs. As a control for these experiments, we show that the level of a control mRNA (36B4) was similar in cells infected with either AdControl or AdWTp53. Thus, the induction of WAF1/Cip1 proteins in cells after infection with AdWTp53 appears to be mediated by an increase in WAF1/Cip1 mRNA, although the possibility of a posttranscriptional regulation of p53 expression cannot be ruled out.

AdWTp53-mediated Apoptosis. To investigate whether the mechanisms of AdWTp53-mediated inhibition of cell growth involved programmed cell death (apoptosis), the effect of AdWTp53 on nucleosomal DNA fragmentation was examined after infection of MDA-MB-231 cells (which express endogenous mutant p53), H-358 cells (which are null for p53), and in MCF-7 and NMECs (both of which express endogenous wild-type p53). As shown in Fig. 6, 24 h after exposure of MDA-MB-231 cells to 50 pfu/cell of AdWTp53, several lower molecular weight DNA bands (DNA laddering of approximately 145 bp) in the range of 145-1050 bp were observed, which are characteristic of cells undergoing apoptosis. Similar results were observed following AdWTp53 infection of H-358 cells (data not shown). In contrast, exposure of MDA-MB-231 cells to AdControl or mock infection of these cells produced no detectable DNA laddering (Fig. 6). Exposure of MCF-7 cells or NMECs to AdWTp53 at 50 pfu/cell or MCF-7 cells to 1000 pfu/cell also did not show any DNA laddering (data not shown). Similarly, when MDA-MB-231, H-358, and MCF-7 cells were infected with AdWTp53 for 24 h, followed by the detection of apoptotic cells by an *in situ* apoptosis detection deoxynucleotidyl transferase end labeling, while MDA-MB-231 and H-358 cells appeared to show a significant number of fluorescent apoptotic cells, no apoptotic cells were present in MCF-7 cell population (data not shown). These results indicate that tumor cells null for *p53* or expressing an endogenous mutant *p53* undergo apoptosis following exposure to AdWTp53, whereas tumor cells or normal cells expressing wild-type p53 are resistant to apoptosis.

DISCUSSION

We have constructed an adenovirus vector expressing a human wild-type p53 protein (AdWTp53) that produces high levels of p53 protein when introduced into normal and malignant human mammary epithelial cells. The rapid induction of wild-type p53 protein following infection of cells with AdWTp53 provided an opportunity to study the biological effects of p53 in cells which differ in their expression of the endogenous *p53* gene.

Tumor cells expressing endogenous mutant *p53* or devoid of *p53* expression were more sensitive to AdWTp53-mediated cytotoxicity when compared with tumor or normal cells expressing endogenous wild-type p53. Moreover, overexpression of wild-type p53 induced programmed cell death (apoptosis) of tumor cells devoid of wild-type p53 or expressing endogenous mutant *p53*, but not in tumor or normal cells expressing wild-type p53. There are several possible mechanisms by which high expression of wild-type p53 results in apoptosis in tumor cells devoid of *p53* or expressing mutant *p53*, but not in tumor or normal cells expressing wild-type p53. For example, cell killing could be dependent on the amount of p53 produced in different cells, stability of p53 protein in different cells, localization of p53 within the cell and the ability (inability) of p53 protein to interact with other cellular factors, and downstream signal transduction pathway. Moreover, as previously suggested (39) a translational modificaton of p53 may play a role in p53-induced apoptosis.

To understand the differential effects of wild-type p53 overexpression in cells with a different intrinsic p53 status, we investigated the expression of two proteins that are known to be regulated by wild-type p53, WAF1/Cip1 and mdm2. AdWTp53-mediated cytotoxic effects appeared to be associated with the high induction of WAF1/Cip1. The *WAF1/Cip1* gene has been shown to bind to cellular cyclin-dependent kinases and thereby inhibit their function (34–36). This inhibition is manifested in turn by a decrease in the level of phosphorylation of the Rb protein (40). Preliminary work in our laboratory has suggested that AdWTp53-mediated p53 protein induction in cells devoid of *p53* or expressing mutant *p53* is associated with dephosphorylation of the Rb protein. On the other hand, in cells expressing wild-type p53, the phosphorylated form of the Rb protein was still present after infection with AdWTp53 (data not shown). Thus, marked induction of WAF1/Cip1 expression after infection with AdWTp53 in turn is associated with an inhibition of the phosphorylation of the Rb protein.

Exposure of cells to DNA-damaging agents such as radiation resulted in apoptosis in normal thymocytes or other cells expressing wild-type p53 (6, 7, 41), while thymocytes and other cancer cells null for *p53* or containing endogenous mutant *p53* were resistant to radiation-mediated cell death (41, 42). The effects of radiation-induced p53 were suggested to be mediated by WAF1/Cip1 induction (41). In the current study we observed that high expression of p53 protein by AdWTp53 was associated with a marked induction of WAF1/Cip1 RNA and protein. Although both radiation and AdWTp53 can induce WAF1/Cip1 protein, whether WAF1/Cip1 is directly responsible for apoptosis is not known. The possibility of a signal transduction agent other than WAF1/Cip1 that mediates the induction of apoptosis in cells by p53 is also possible. Our future experiments will be directed toward understanding the role of WAF1/Cip1 and other signal transduction agents in AdWTp53-mediated apoptosis.

The presence of mutated *p53* is widespread in different human cancers. Thus, reconstituting tumor suppressor *p53* gene expression by adenovirus vectors is an attractive strategy for cancer gene therapy. Since adenovirus enters human epithelial cells with an efficient low pH endosome-mediated endocytosis (19, 20), tumors of mammary epithelial origin will be especially amenable to treatment by AdWTp53. Previous studies by Liu *et al.* (43) have shown that adenovirus-mediated expression of *p53* could cause killing of cells derived from head and neck tumors and of lung cancer cells in the presence of *cis*-platinum (44). As shown here, normal mammary epithelial cells are resistant to apoptosis by AdWTp53, while the tumor cells null for *p53* or expressing mutant *p53* readily undergo apoptosis. Thus, there is a specificity to AdWTp53-mediated killing of tumor cells, lending further support to these vectors for gene therapy of cancer.

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REFERENCES

1. Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F. C., Weston, A., Modali, R., Harris, C. C., and Vogelstein, B. Mutations in the *p53* gene occur in diverse human tumor types. Nature (Lond.), *342*: 705–708, 1989.

2. Takahashi, T., Nau, M. M., Chiba, I., Birrer, M. J., Rosenberg, R. K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A. F., and Minna, J. D. p53: a frequent target for genetic abnormalities in lung cancer. Science (Washington DC), *246*: 491–494, 1989.

3. Srivastava, S., Zou, Z., Pirollo, K., Blattner, W., and Chang, E. H. Germ-line transmition of a mutated *p53* gene in a cancer-prone family with Li-Fraumeni syndrome. Nature (Lond.), *348*: 747–749, 1990.

4. Chen, P., Chen, Y., Bookstein, R., and Lee, W. Genetic mechanisms of tumor suppression by the human *p53* gene. Science (Washington DC), *250*: 1576–1579, 1990.

5. Gottesman, M. M. Report of a meeting: molecular basis of cancer therapy. J. Natl. Cancer Inst., *86*: 1277–1285, 1994.

6. Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L., and Wyllie, A. H. Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature (Lond.), *362*: 849–852, 1993.

7. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature (Lond.), *362*: 847–849, 1993.

8. Dulić, V., Kaufmann, W. K., Wilson, S. J., Tlsty, T. D., Lees, E., Harper, J. W., Elledge, S. J., and Reed, S. I. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G$_1$ arrest. Cell, *76*: 1013–1023, 1994.

9. Nelson, W. G., and Kastan, M. B. DNA strand brakes: the DNA template alterations that trigger p53-dependent DNA damage response pathways. Mol. Cell. Biol., *14*: 1815–1823, 1994.

10. Michalovitz, D., Halevy, O., and Oren, M. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. Cell, *62*: 671–680, 1990.

11. Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A., and Oren, M. Wild-type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6. Nature (Lond.), *352:* 345–347, 1991.

12. Chiou, S., Rao, L., and White, E. Bcl-2 blocks p53-dependent apoptosis. Mol. Cell. Biol., *14:* 2556–2563, 1994.

13. Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. Nature (Lond.), *356:* 215–221, 1992.

14. Shaw, P., Bovey, R., Tardy, S., Sahli, R., Sordat, B., and Costa, J. Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc. Natl. Acad. Sci. USA, *89:* 4495–4499, 1992.

15. Lowe, S. W., Jacks, T., Housman, D. E., and Ruley, H. E. Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells. Proc. Natl. Acad. Sci. USA, *91:* 2026–2030, 1994.

16. Casey, G., Lo-Hsueh, M., Lopez, M. E., Vogelstein, B., and Stanbridge, E. J. Growth suppression of human breast cancer cells by the introduction of a wild-type *p53* gene. Oncogene, *6:* 1807–1811, 1991.

17. Ginsberg, H. S. Adenoviruses. *In:* R. Dulbecco and H. S. Ginsberg (eds.), Virology, pp. 147–160. Philadelpia: J. B. Lippincott Co., 1988.

18. Graham, F. L., and Prevec, L. Manipulation of adenovirus vectors. *In:* E. J. Murray (ed.), Gene Transfer and Expression Protocols, pp. 109–128. Clifton, NJ: Humana Press, 1991.

19. Seth, P., Fitzgerald, D., Ginsberg, H., Willingham, M., and Pastan, I. Pathway of adenovirus entry into cells. *In:* R. Crowell and K. Lonberg-Holm (eds.), Virus Attachment and Entry into Cells, pp. 191–195. Washington, DC: American Society for Microbiology, 1986.

20. Seth, P., Rosenfeld, M., Higginbotham, J., and Crystal, R. G. Mechanism of enhancement of DNA expression consequent to cointernalization of a replication-deficient adenovirus and unmodified plasmid DNA. J. Virol., *68:* 933–940, 1994.

21. Jones, N., and Shenk, T. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell, *17:* 683–689, 1979.

22. Rosenfeld, M. A., Chu, C., Seth, P., Danel, C., Banks, T., Yoneyama, K., Yoshimura, K., and Crystal, R. G. Gene transfer to freshly isolated human respiratory epithelial cells *in vitro* using a replication-deficient adenovirus containing the human cystic fibrosis transmembrane conductance regulator cDNA. Hum. Gene Ther., *5:* 331–342, 1994.

23. Zakut-Houri, R., Bienz-Tadmor, B., Givol, D., and Oren, M. Human p53 cellular tumor antigen: cDNA sequence and expression in COS cells. EMBO J., *4:* 1251–1255, 1985.

24. McGrory, W. J., Bautista, D. S., and Graham, F. L. A simple technique for the rescue of early region I mutation into infectious human adenovirus type 5. Virology, *163:* 614–617, 1988.

25. Gilardi, P., Courtney, M., Pavirani, A., and Perricaudet, M. Expression of human alpha1-antitrypsin using a recombinant adenovirus vector. FEBS Lett., *267:* 60–62, 1990.

26. Katayose, D., Ohe, M., Yamauchi, K., Ogata, M., Shirato, K., Fujita, H., Shibahara, S., and Takishima, T. Increased expression of PDGF A- and B-chain genes in rat lungs with hypoxic pulmonary hypertension. Am. J. Physiol., *264:* L100–L106, 1993.

27. Stratford-Perricaudet, L. D., Makeh, I., Perricaudet, M., and Briand, P. J. Widespread long-term gene transfer to mouse skeletal muscles and heart. J. Clin. Invest., *90:* 626–630, 1992.

28. Fairchild, C. R., Ivy, S. P., Kao-Shan, C., Whang-Peng, J., Rosen, N., Israel, M. A., Melera, P. W., Cowan, K. H., and Goldsmith, M. E. Isolation of amplified and overexpressed DNA sequences from adriamycin-resistant human breast cancer cells. Cancer Res., *47:* 5141–5148, 1987.

29. Gudas, J. M., Oka, M., Diella, F., Trepel, J., and Cowan, K. H. Expression of wild-type p53 during the cell cycle in normal human mammary epithelial cells. Cell Growth & Differ., *5:* 295–304, 1994.

30. Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., and Boyd, M. R. New colorimetric cytotoxicity assay for anticancer drug screening. J. Natl. Cancer Inst., *82:* 1107–1112, 1990.

31. Seth, P., Fitzgerald, D., Ginsberg, H., Willingham, M., and Pastan, I. Evidence that the penton base of adenovirus is involved in potentiation of toxicity of pseudomonous exotoxin conjugated to epidermal growth factor. Mol. Cell. Biol., *4:* 1528–1533, 1984.

32. Bartek, J., Iggo, R., Gannon, J., and Lane, D. P. Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. Oncogene, *5:* 893–899, 1990.

33. Haldar, S., Negrini, M., Monne, M., Sabbioni, S., and Croce, C. M. Down-regulation of *bcl-2* by *p53* in breast cancer cells. Cancer Res., *54:* 2095–2097, 1994.

34. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. p21 is a universal inhibitor of cyclin kinase. Nature (Lond.), *366:* 701–704, 1993.

35. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. Cell, *75:* 817–825, 1993.

36. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. The p21 Cdk-interacting Cip1 is a potential inhibitor of G1 cyclin-dependent kinases. Cell, *75:* 805–816, 1993.

37. Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. L. The *mdm-2* oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell, *69:* 1237–1245, 1992.

38. Ullrich, S. J., Anderson, C. W., Mercer, W. E., and Appella, E. The p53 tumor suppressor protein, a modulator of cell proliferation. J. Biol. Chem., *267:* 15259–15262, 1992.

39. Caelles, C., Helmberg, A., and Karin, M. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. Nature (Lond.), *370:* 220–223, 1994.

40. Ewen, M. E. The cell cycle and the retinoblastoma protein family. Cancer Metastasis Rev., *13:* 45–66, 1994.

41. El-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., Pietenpol, J. A., Burrell, M., Hill, D. E., Wang, Y., Wiman, K. G., Mercer, W. E., Kastan, M. B., Kohn, K. W., Elledge, S. J., Kinzler, K. W., and Vogelstein, B. *WAF1/CIP1* is induced in *p53*-mediated G_1 arrest and apoptosis. Cancer Res., *54:* 1169–1174, 1994.

42. McIlwrath, A. J., Vasey, P. A., Ross, G. M., and Brown, R. Cell cycle arrests and radiosensitivity of human tumor cell lines: dependence on wild-type p53 for radiosensitivity. Cancer Res., *54:* 3718–3722, 1994.

43. Liu, T., Zhang, W., Taylor, D. L., Roth, J. A., Goepfert, H., and Clayman, G. L. Growth suppression of human head and neck cancer cells by the introduction of a wild-type *p53* gene via recombinant adenovirus. Cancer Res., *54:* 3662–3667, 1994.

44. Fujiwara, T., Grimm, E. A., Mukhopadhyay, T., Zhang, W., Owen-Schaub, L. B., and Roth, J. A. Induction of chemosensitivity in human lung cancer cells *in vivo* by adenovirus-mediated transfer of the wild-type *p53* gene. Cancer Res., *54:* 2287–2291, 1994.

RECOMBINANT ADENOVIRUS VECTOR EXPRESSING WILD-TYPE p53 IS A POTENT INHIBITOR OF PROSTATE CANCER CELL PROLIFERATION*

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ABSTRACT—**Objectives.** A recombinant adenovirus vector (AdWTp53) expressing wild-type p53 was evaluated for its cell growth inhibitory effects on metastatic human prostate cancer cells.

Methods. Human prostate cancer cells LNCaP, DU145, PC3, 1LN, and DUPro-1 were infected with AdWTp53 vector and expression of exogenous p53 in these cells was analyzed by immunoprecipitation and western blot assays. The cell growth inhibitory effects of AdWTp53 were determined by counting cell number on a hemocytometer or by crystal violet staining of cells after infection with AdWTp53. The p53-regulated gene WAF1 and DNA fragmentation were also analyzed in prostate cancer cells infected with AdWTp53.

Results. High levels of the AdWTp53 vector-derived p53 protein were present in metastatic prostate cancer cells, and the p53-regulated gene WAF1 was induced in these cells. Infection of these tumor cell lines with AdWTp53 vector resulted in severe growth inhibition and cell death in comparison to untreated or control adenovirus vector–infected cells. Furthermore, fragmentation of genomic DNA, a property associated with apoptosis, was also observed in prostate cancer cells infected with AdWTp53.

Conclusions. AdWTp53 vector exhibited a potent inhibitory effect on the growth of all of human metastatic prostate cancer cells, and both cytostatic and cytotoxic effects of AdWTp53 were observed. The induction of p53-regulated gene WAF1 in AdWTp53-infected prostate cancer cells suggests the involvement of cellular p53 pathway in the cell growth inhibition. These results provide a molecular basis for further evaluation of antitumorigenic effects of AdWTp53 vector in animal models of prostate cancer. UROLOGY® **46**: 843–848, 1995.

The tumor suppressor gene p53 is one of the most frequently altered genes detected in human tumors. Both somatic and germline mutations of p53 have been reported.[1,2] Numerous early studies of p53 alterations in prostate cancers have revealed a low incidence (10% to 20% of specimens analyzed) of p53 mutations in prostate cancers.[3,4] However, more recent studies suggest that p53 alterations may be frequent in a subset of prostate cancers, especially in hormone refractory disease.[5-9] The tumor suppressor activity of the p53 gene has been demonstrated for diverse tumor cell types,[1] including prostate tumor cells.[10] The biochemical/biologic functions of the native p53 protein include target gene transactivation, cell cycle checkpoint control, and programmed cell death.[1,11,12] One or more of these functions are known to be deregulated in tumor cells.[12] Thus, correction of p53 dysfunctions in human cancers may have widespread application in anticancer therapy. Adenovirus vectors containing potential therapeutic genes are currently being evaluated for their utility in gene therapy of cancer and other human diseases.[13] Adenovirus-based vectors are internalized into epithelial cells with an efficient receptor-mediated endocytosis, can be grown to high titers, and express a transgene to high levels without integrating into the cellular genome.[13-15] Recent reports of antiproliferative and antitumorigenic effects of a recombinant adenovirus vector expressing wild-type (wt) p53 in nonsmall cell lung cancer cells[16] and head and neck cancer cells[17] in vitro and in nude mice provide early insights

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regarding the potential of adenovirus-based vectors in cancer gene therapy. We have also recently described the construction of the AdWTp53 and its antiproliferative effects on breast cancer cells.[18] In this report, we have evaluated the biologic effects of the AdWTp53 vector on human metastatic prostate cancer cells and describe a potent cell growth inhibitory effect of this vector for several metastatic prostate cancer cell lines. Induction of the p53-regulated gene WAF1 and DNA fragmentation in prostate cancer cells infected with AdWTp53 suggested the activation of cellular p53 pathway in those cells.

MATERIAL AND METHODS

Cell Culture

LNCaP, DU145, and PC3 cells, derived from metastatic lesions of human prostate cancer, and MCF7 derived from a human breast adenocarcinoma were obtained from American Type Culture Collection (Rockville, Md). 1LN PC3-1A (1LN) cells, nude mouse metastatic variant of PC3 cells,[19] and DuPro-1 cells, derived from the athymic nude mice xenograft of a lymph node metastasis of the prostate adenocarcinoma,[20] were kindly provided by Dr. David Paulson (Duke University Medical Center, Durham, NC). Normal human foreskin fibroblast cells, H500, have been described elsewhere.[21]

AdWTp53 Expression Vector

The construction of the AdWTp53 recombinant adenovirus expressing human wt p53 has been described elsewhere.[18] In brief, homologous recombination between a shuttle vector containing human wt p53 cDNA downstream of a cytomegalovirus promoter and the adenovirus genome cloned in a plasmid pJM17 resulted in a recombinant adenovirus in which E1 region was replaced by wt p53 expression cassette. The control adenovirus dl 312 (C-Ad) lacked the E1 region and wt p53 expression cassette.

Metabolic Labeling, Immunoprecipitation, and Western Blotting

Cells were labeled with Trans[^{35}S]-label,[22] lysed in lysis buffer,[21] and processed for immunoprecipitation with anti-p53 antibody, PAb1801, or anti p21/WAF1, Ab-1 antibody (Oncogene Science, Uniondale, NY). Immunoprecipitates were analyzed for p53 protein on sodium dodecyl sulfate (SDS)-8% polyacrylamide gels[22] and for WAF1/CIP1 p21 protein on SDS-14% polyacrylamide gels.

For the immunoblotting procedure, cell lysates equivalent to 100 μg total protein were electrophoresed on SDS-14% polyacrylamide gel followed by electroblotting[23] onto nitrocellulose membranes. p53 and WAF1 proteins on the blot were detected with anti-p53 and anti-WAF1 monoclonal antibodies, respectively, and utilizing [^{125}I]-protein A or alkaline phosphatase–based detection system.

Cell Growth Inhibition Assay

Exponentially growing cells were seeded at 0.5 to 1 × 10^5 cells/well in six-well dishes. Twenty-four hours later growth medium was replaced with 2 mL of growth medium containing 2% fetal bovine serum and desired dosages (plaque-forming unit [pfu]/cell) of AdWTp53 or C-Ad. After 2 hours of incubation at 37°C in a carbon dioxide incubator, 3 mL of growth media with 10% fetal bovine serum was added and growth of cells was monitored every other day. Five days postinfection or as indicated, cells attached to the culture dish were fixed and stained with crystal violet. Cell growth inhibition was also monitored by counting cells on the hemocytometer. To determine number of live and dead cells, cells were treated with trypan blue and cells showing the uptake of the dye were interpreted as nonviable.

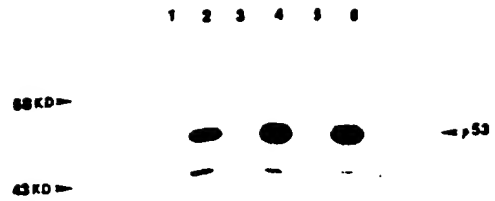

FIGURE 1. *Detection of p53 protein in AdWTp53-infected prostate cancer cells. LNCaP (lanes 1 and 2), DU145 (lanes 3 and 4), and PC3 (lanes 5 and 6) prostate cancer cells (2 × 10^6 cells) were infected with 20 pfu/cell of control adenovirus (lanes 1, 3, and 5) or AdWTp53 (lanes 2, 4, and 6) for 20 hours. Cells were labeled with Trans[^{35}S]-label and cell lysates equivalent to 4 × 10^6 counts per minute of trichloroacetic acid precipitable protein were immunoprecipitated with anti-p53 antibody and analyzed by sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis as described under "Material and Methods." Arrow with p53 indicates AdWTp53-derived wt p53 in LNCaP (lane 2), DU145 (lane 4), and PC3 (lane 6) cells.*

DoxoRubicin-Mediated Induction of WAF1/CIP1 Protein

Cells, 2 × 10^6 cells (LNCaP, DU145, PC3, or H500) were plated in 100-mm dishes and were treated with 0.2 μg/mL of doxorubicin (Adriamycin) or same volume of phosphate-buffered saline for 21 hours followed by preparation of cell lysates and immunoblotting to detect WAF1/CIP1 protein.

DNA Fragmentation Analysis

Total genomic DNA was extracted from untreated or AdWTp53 infected DU145 and LNCaP cells and analyzed by agarose gel electrophoresis on 2.5% (w/v) agarose gels and ethidium bromide staining.

RESULTS

AdWTp53-Mediated Expression of p53 in Prostate Cancer Cells

We first determined if AdWTp53 vector-derived wt p53 protein was expressed in prostate cancer cells. Prostate cancer cell lines DU145, PC3, and LNCaP were infected with 20 pfu/cell of AdWTp53 vector or C-Ad for 20 to 24 hours followed by metabolic labeling of cells with Trans[^{35}S]-label and immunoprecipitation by anti-p53 monoclonal antibody PAb1801. As shown in Figure 1, high levels of p53 protein were detected in DU145, PC3, and LNCaP cells infected with AdWTp53. No p53 was detectable in C-Ad–infected cells under similar conditions. However, longer exposure of the

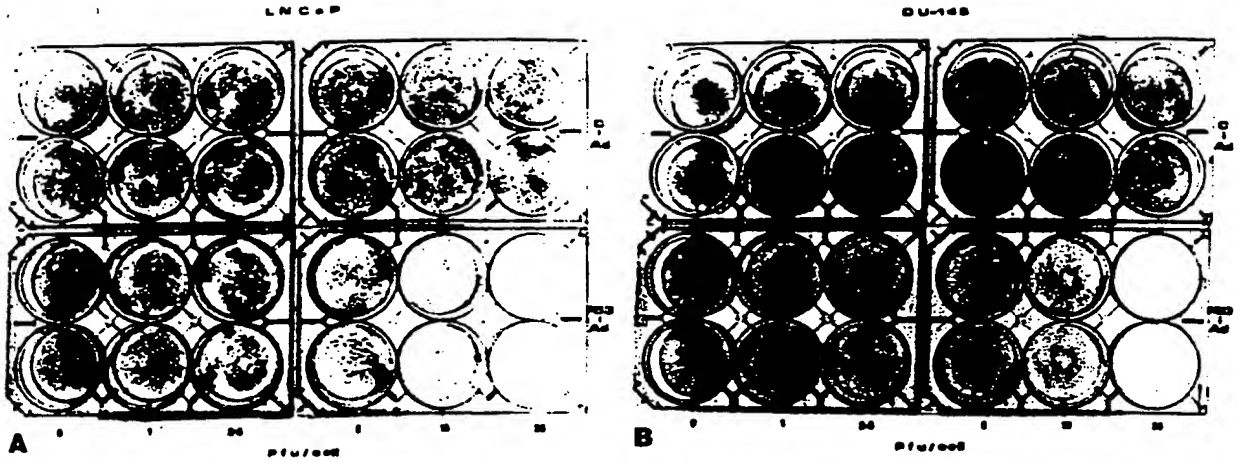

FIGURE 2. *AdWTp53-mediated inhibition of prostate cancer cell proliferation. LNCaP (A) and DU145 (B) cells were treated with different dosages (plaque-forming unit/cell) of control adenovirus or AdWTp53 shown here as C-Ad or p53-Ad, respectively. The cells were fixed and stained with crystal violet after 96 hours of the infection. Violet stain shown in black here represents the presence of cells attached to tissue culture dish.*

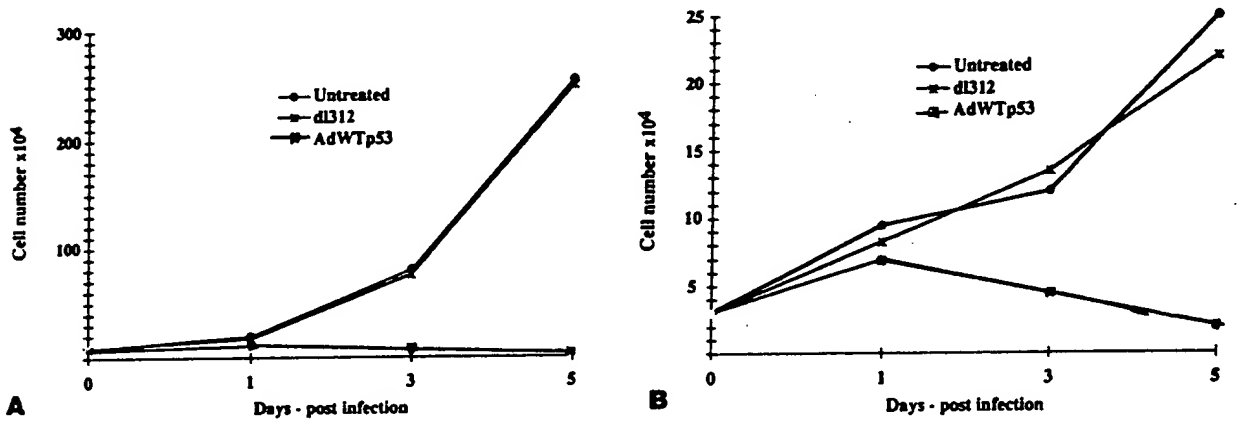

FIGURE 3. *Time course of AdWTp53-mediated inhibition of prostate cancer cell growth. DU145 (A) and LNCaP (B) cells infected with control adenovirus: dl 312 or AdWTp53 vector were analyzed for their growth at different times after infection. Uninfected cells were also analyzed in parallel. The cell growth was monitored by counting cells on the hemocytometer at time 0 and at indicated times postinfection.*

gels to x-ray film revealed faint endogenous p53 bands in C-Ad–infected DU145 and LNCaP cells (data not shown).

Growth Inhibitory Effects of AdWTp53 on Prostate Cancer Cells

To analyze for effects of AdWTp53 on growth of prostate cancer cells, we infected DU145 and LNCaP cells with varying dosage of AdWTp53 or a control adenovirus vector. AdWTp53-infected cells began to exhibit growth inhibition at 2.5 plaque forming unit (pfu)/cell with a marked growth inhibition (more than 90%) between 10 to 20 pfu/cell (Fig. 2A, B). Similar results were also obtained from two other metastatic prostate cancer cells: 1LN and DUPro-1 (data not shown). Although there was some inhibitory effect (10% to 15%) on cell growth at or above 20 pfu/cell of C-Ad, several independent experiments showed a dramatic growth inhibition (95% to 99%) of prostate cancer cells after 5 to 6 days in the presence of 10 to 20 pfu/cell of AdWTp53. We also did not observe any cytotoxic or growth inhibitory effects of AdWTp53 on human fibroblast cells. Although level of p53 in AdWTp53-infected H500 was not as high as in prostate cancer cells, it was at least fivefold over endogenous levels (data not shown). Since several previous studies did not observe cell growth inhibitory effects of exogenous p53 in tumor cells that already contained endogenous wt p53,[24,25] the inhibitory effects of AdWTp53 on LNCaP cells containing endogenous wt p53 was unexpected. However, in agreement with the previous observations,[18] we also did not detect a growth inhibitory effect of AdWTp53 on breast cancer cells, MCF7 containing endogenous wt p53 (data not shown). The kinetics of cell growth inhibition in response to AdWTp53 were further evaluated for two representative cell

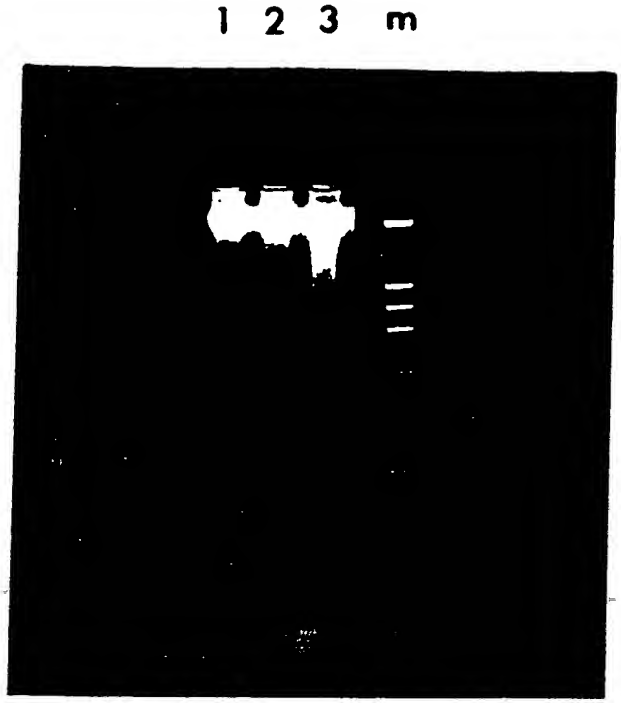

FIGURE 4. *Analysis of genomic DNA from LNCaP cells infected with AdWTp53. Total genomic DNA was isolated from untreated LNCaP cells (lane 1), cells infected with 20 plaque-forming unit/cell of control adenovirus (lane 2), or AdWTp53 (lane 3) and analyzed on 2.5% agarose gel followed by ethidium bromide staining. m:λ HindIII + 1 × HaeIII DNA molecular weight marker.*

lines: DU145 and LNCaP. As shown in Figure 3, DU145 cells exhibited almost complete inhibition by day 3, whereas LNCaP (data not shown) cells exhibited 95% inhibition by day 5. Furthermore, infection of 1×10^6 cells of DU145 or LNCaP with 20 pfu/cell of AdWTp53 resulted in a large number of floating cells (representing 50% to 60% of total cells) within 48 to 50 hours, and more than 95% of these floating cells were nonviable as determined by trypan blue exclusion assay (data not shown). Additionally, we analyzed the genomic DNA from AdWTp53-infected LNCaP or DU145 cells for DNA fragmentation, a property known to be associated with programmed cell death. The analysis of genomic DNA from LNCaP (Fig. 4) and DU145 (data not shown) cells revealed DNA fragmentation in the AdWTp53-treated cells, whereas untreated or control adenovirus injected cells did not show any evidence of detectable DNA fragmentation.

Induction of WAF-1/CIP-1 by AdWTp53 or Doxorubicin in Prostate Cancer Cells

The expression of WAF1/CIP1 encoded p21 protein, an inhibitor of the cyclin-dependent kinases, has been shown to be directly up regulated by the wt p53.[26] Although WAF1 p21 protein was not readily detectable in PC3, LNCaP and DU145 cells (Fig. 5A) similar steady-state level of WAF1 RNA was detected in these cells (data not shown). All three prostate cancer cell lines, PC3, DU145, and LNCaP infected with AdWTp53, exhibited increased levels of WAF1/CIP1 p21 protein in comparison to the respective cell lines infected with C-Ad (Fig. 5A). To ascertain that LNCaP cells used in this study were not variant and did contain endogenous wt p53 function, we assayed the induction of WAF1 protein in LNCaP cells in response to doxorubicin, a chemotherapeutic agent reported to induce WAF1 only in those cells that contain wt p53.[27] As shown in Figure 5B, doxorubicin resulted in the induction of WAF1 p21

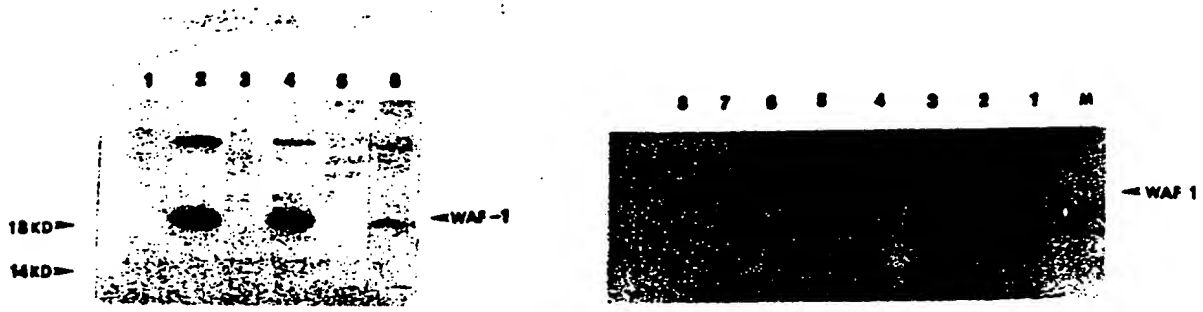

FIGURE 5. *Analysis of WAF1 in prostate cancer cells. (A) WAF1/CIP1 induction in AdWTp53 infected cells: LNCaP (lanes 1 and 2), DU145 (lanes 3 and 4), and PC3 (lanes 5 and 6). Lanes 1, 3, and 5: cells infected with control adenovirus vector; lanes 2, 4, and 6: cells infected with AdWTp53 vector. Immunoprecipitation of WAF1 protein was performed as described under "Material and Methods." (B) Effect of doxorubicin treatment on the induction of WAF1/CIP1: prostate cancer cells (2×10^6); LNCaP (lanes 1, 2), PC3 (lanes 3, 4), DU145 (lanes 5, 6), and human fibroblast cells (2×10^6); H500 (lanes 7, 8) were treated with doxorubicin (lanes 2, 4, 6, and 8) or phosphate-buffered saline (lanes 1, 3, 5, and 7). Immunoblotting of WAF1 protein was performed on cell lysates and described under "Material and Methods."*

protein in LNCaP cells (lane 2) and in normal human fibroblasts, H500 (lane 8). However, WAF1 p21 protein induction was not seen in PC3 (lane 4) or DU145 (lane 6) cells.

COMMENT

Gene therapeutic strategies for human prostate cancer encompass different approaches and represent an early phase of research in this direction. There are few reports that have described promising results with gene/immunotherapy of metastatic prostate cancer in an animal model system.[28,29] In these studies, genetically engineered prostate cancer cells producing recombinant cytokines provided effective therapy for prostate cancer in the Dunning rat prostate carcinoma model. The tumor vaccine approach described before is promising; however, the technology involved is very complicated and the major limitations precluding its wider application could be the availability and engineering of prostate cancer cells from the same patient in which it is to be utilized as vaccine. Other emerging molecular approaches for anticancer therapy include corrective gene therapy to correct a known molecular defect in cancer cells, for example, inhibition of an activated oncogene by antisense RNA/oligonucleotide or reconstitution of a defective or absent tumor suppressor gene (TSG) function by reintroduction of the normal copy of that TSG. The antiproliferative or antitumorigenic properties of the TSGs strongly suggest their therapeutic potential. However, the current challenges include the generation of efficient vectors for these potentially therapeutic genes and their delivery to cancer cells. Antiproliferative or antitumorigenic effects of the TSG p53 have been described for diverse types of tumor cells.[1] In this report, we have characterized the cell growth inhibitory effects of a recombinant adenovirus vector expressing high levels of wt p53 in human metastatic prostate cancer cells.

The expression of wt p53 is achieved at a very high level (at least 100-fold in comparison to the endogenous p53 bands seen in LNCaP or DU145 cells) in AdWTp53-infected prostate cancer cells. In our experiments, all three established prostate cancer cell lines, DU145, LNCaP, and PC3, as well as two other metastatic prostate cancer cells, 1LN and DUPro-1, were similarly growth inhibited by AdWTp53 vector. Time course study of the infection of prostate cancer cells, presence of nonviable floating cells, and DNA fragmentation in AdWTp53-infected LNCaP and DU145 cells suggested a combination of cell growth arrest and cell death effects, both of which are known to be associated with wt p53 cellular function.[1]

The inhibitory effects of AdWTp53 on LNCaP cells containing wt p53 were unexpected. We, therefore, have further assessed the status of p53 in LNCaP cells by utilizing an assay in which induction of the WAF1/CIP1 gene in response to DNA damaging agents is tightly correlated with the presence of functional wt p53 in cells.[27] The induction of WAF1 in response to doxorubicin in LNCaP cells but not in PC3 or DU145 strongly suggests the presence of wt p53 in LNCaP cells used in this study. Although a majority of reports have noticed growth inhibitory effects of wt p53 on tumor cells with mutant or no p53,[1,24,25] there are some studies that have also described growth inhibitory effects of wt p53 on tumor cells with endogenous wt p53.[17,18,30] However, it is possible that some as yet unknown function of p53 is defective in LNCaP cells studied here and further study is warranted to understand the mechanisms of inhibitory effects of AdWTp53 on LNCaP cells.

Our studies along with the earlier report describing p53 tumor suppressor effects on two prostate cancer cell lines, PC3 and TSU-PR1,[10] demonstrate that all six metastatic prostate cancer cell lines tested so far are growth inhibited in response to the overexpression of exogenous wt p53. Our studies with AdWTp53 vector provide support for earlier observations[10] and, more importantly, provide a wt p53 expression vector with a potential for future applications in gene therapy–related experiments. Recent reports have also described the gene therapy potential of recombinant adenovirus vectors expressing wt p53 in animal models of lung[16] and head and neck[17] cancers. A recent study has described an intriguing result in which an adenovirus-p53 expression vector did not inhibit the in vitro growth of a metastatic variant of LNCaP cells; however, the growth of these cells was inhibited in vivo.[31] In contrast, adenovirus-p53 expression vector utilized in our study exhibited a potent inhibitory effect on all the metastatic human prostate cancer cell lines tested. Another report has recently evaluated the therapeutic efficacy of a recombinant adenovirus vector expressing wt p53 in a mouse prostate reconstitution model.[32] Although the primary tumors derived from mouse prostate cancer cells harboring homozygous p53 mutation and exogenous *ras* and *myc* oncogenes did not show reduction in size following the injection of adenovirus vector expressing wt p53, there was a marked suppression of metastatic lesions.[32] The mechanism of inhibition of prostate cancer cell growth in response to AdWTp53 vector appears to involve cellular p53 pathway as evident from the up regulation of WAF1/CIP1 protein and DNA fragmentation in AdWTp53-infected prostate cancer cells noted in our study. Our preliminary results from intratumoral injections of the AdWTp53 have also shown inhibition of tumor progression

of DU145- and PC3-derived tumors in nude mice (data not shown). Additional studies utilizing animal models of prostate cancer will further characterize the in vivo antitumorigenic effects of the AdWTp53 vector.

ACKNOWLEDGMENT. To Shirley Craig for the preparation of the manuscript.

REFERENCES

1. Greenblatt MS, Bennett WP, Hollstein M, and Harris CC: Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 54: 4855–4878, 1994.

2. Malkin D: p53 and the Li-Fraumeni syndrome. Biochim Biophys Acta 1198: 197–213, 1994.

3. Isaacs WB, Bova GS, Morton RA, Bussemakers MJ, Brooks JD, and Ewing CM: Molecular biology of prostate cancer. Semin Oncol 21: 514–521, 1994.

4. Moul JW, Gaddipati J, and Srivastava S: Molecular biology of prostate cancer: oncogenes and tumor suppressor genes, in Dawson NA and Vogelzang N (Eds.): *Current Clinical Oncology: Prostate Cancer*. New York, Wiley-Liss Publications, 1994, pp 19–46.

5. Bookstein R, MacGrogan D, Hilsenbeck SG, Sharkey F, and Allred DC: p53 is mutated in a subset of advanced-stage human prostate cancers. Cancer Res 53: 3369–3373, 1993.

6. Navone NM, Troncoso P, Pisters LL, Goodrow TL, Palmer JL, Nichols WW, von Eschenbach AC, and Conti CJ: p53 protein accumulation and gene mutation in the progression of human prostate carcinoma. J Natl Cancer Inst 85: 1657–1669, 1993.

7. Aprikian AG, Sarkis AS, Fair WR, Zhang ZF, Fuks Z, and Cordon-Cardo C: Immunohistochemical determination of p53 protein nuclear accumulation in prostatic adenocarcinoma. J Urol 151: 1276–1280, 1994.

8. Berner A, Nesland JM, Waehre H, Silde J, and Fossa SD: Hormone resistant prostatic adenocarcinoma. An evaluation of prognostic factors in pre- and post-treatment specimens. Br J Cancer 68: 380–384, 1993.

9. Heidenberg H, Sesterhenn IA, Gaddipati J, Weghorst M, Buzard GS, Moul JW, and Srivastava S: Alteration of the tumor suppressor gene p53 in a high fraction of hormone refractory prostate cancer. J Urol 154: 414–421, 1995.

10. Isaacs WB, Carter BS, and Ewing CM: Wild-type p53 suppresses growth of human prostate cancer cells containing mutant p53 alleles. Cancer Res 51: 4716–4720, 1991.

11. Vogelstein B, and Kinzler KW: p53 function and dysfunction. Cell 70: 523–526, 1992.

12. Hartwell LH, and Kastan MB: Cell cycle control and cancer. Science 266: 1821–1828, 1994.

13. Kozarsky KF, and Wilson JM: Gene therapy: adenovirus vectors. Curr Opin Genet Dev 3: 499–503, 1993.

14. Seth P, Fitzgerald D, Ginsberg H, Willingham M, and Pastan I: Pathway of adenovirus entry into cells, in Crowell R, and Lonberg-Holm K (Eds): *Virus Attachment and Entry into Cells*. American Society for Microbiology, Washington, DC, 1986, pp 191–195.

15. Rosenfeld MA, Chu CS, Seth P, Danel C, Banks T, Yoneyama K, Yoshimura K, and Crystal RG: Gene transfer to freshly isolated human respiratory epithelial cells in vitro using a replication deficient adenovirus containing the human cystic fibrosis transmembrane conductance regulator cDNA. Hum Gene Ther 5: 331–342, 1994.

16. Fujiwara T, Grimm EA, Mukhopadhyay T, Zhang WW, Owen-Schaub LB, and Roth JA: Induction of chemosensitivity in human lung cancer cells in vivo by adenovirus-mediated transfer of wild-type p53 gene. Cancer Res 54: 2287–2291, 1994.

17. Clayman GL, el-Naggar AK, Roth JA, Zhang WW, Goepfert H, Taylor DL, and Liu TJ: In vivo molecular therapy with p53 adenovirus for microscopic residual head and neck squamous carcinoma. Cancer Res 55: 1–6, 1995.

18. Katayose D, Gudas J, Nguyen H, Srivastava S, Cowan KH, and Seth P: Cytotoxic effects of adenovirus mediated wild type p53 protein expression in normal and tumor mammary epithelial cells. Clin Cancer Res 1: 889–897, 1995.

19. Ware JL, Paulson DF, Mickey GH, and Webb KS: Spontaneous metastasis of cells of the human prostate carcinoma cell line PC-3 in athymic nude mice. J Urol 128: 1064–1067, 1982.

20. Gingrich JR, Tucker JA, Walther PJ, Day JW, Poulton SH, and Webb KS: Establishment and characterization of a new human prostatic carcinoma cell line (DuPro-1). J Urol 146: 915–919, 1991.

21. Janat MF, Srivastava S, Devadas K, Chin GA, Pirollo KF, Chang EH: Inhibition of retinoblastoma protein phosphorylation by the combined effect of interferon-γ and tumor necrosis factor-∝. Mol Cell Diff 2: 241–253, 1994.

22. Srivastava S, Tong YA, Devadas K, Zou ZQ, Sykes VW, Chen Y, Blattner WA, Pirollo K, and Chang EH: Detection of both mutant and wild-type p53 protein in normal skin fibroblasts and demonstration of a shared "second-hit" on p53 in diverse tumors from a cancer-prone family with Li-Fraumeni syndrome. Oncogene 7: 987–991, 1992.

23. Towbin H, Staehelin T, and Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels into nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76: 4350–4354, 1979.

24. Baker SJ, Markowitz S, Fearon ER, Willson JK, and Vogelstein B: Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 249: 912–915, 1990.

25. Oren M: p53: the ultimate tumor suppressor gene? FASEB J 6: 3169–3176, 1992.

26. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, and Vogelstein B: WAF1, a potential mediator of p53 tumor suppression. Cell 75: 817–825, 1993.

27. el-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang Y, *et al:* WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res 54: 1169–1174, 1994.

28. Sanda MG, Ayyagari SR, Jaffee EM, Epstein JI, Clift SL, Cohen LK, Dranoff G, Pardoll DM, Mulligan RC, and Simons JW: Demonstration of a rational strategy for human prostate cancer gene therapy. J Urol 151: 622–628, 1994.

29. Vieweg J, Rosenthal FM, Bannerji R, Heston WD, Fair WR, Gransbacher B, and Gilboa E: Immunotherapy of prostate cancer in Dunning rat model: use of cytokine gene modified tumor vaccine. Cancer Res 52: 1760–1765, 1994.

30. Cajot JF, Anderson MJ, Lehman TA, Shapiro H, Briggs AA, and Stanbridge EJ: Growth suppression mediated by transfection of p53 in Hut292DM human lung cancer cells expressing endogenous wild type p53 protein. Cancer Res 52: 6956–6960, 1992.

31. Ko AG, Gotoh A, Thalman GN, Cleutjens C, Trapman J, Ling-Jun H, Zhang WW, Chung LK, and Kao C: Gene therapy for treatment of androgen independent and PSA producing human prostate cancer. Basic and Clinical Aspects of Prostate Cancer Meeting, Palm Springs, Abstract C-21, Dec 1994.

32. Sehgal I, Eastham JA, Zhang WW, Hall S, Park SH, Kadmon D, Scardino PT, and Thompson TC: Adenovirus-mediated p53 gene transfer in experimental prostate cancer. Abstract 321, AUA Ninetieth Annual Meeting April 23–28, 1995. J Urol 153: 309A, 1995.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
GARY L. CLAYMAN

Serial No.: 08/758,033

Filed: November 27, 1996

For: METHOD AND COMPOSITION FOR THE DIAGNOSIS AND TREATMENT OF CANCER

Group Art Unit: 1632

Examiner: K. Hauda

Atty. Dkt. No.: INRP:041/HYL

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37 C.F.R. 1.8

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DECLARATION OF DR. GARY L. CLAYMAN UNDER 37 C.F.R. § 1.131

Hon. Assistant Commissioner for Patents
Washington, D.C. 20231

I, Gary L. Clayman, D.D.S., M.D., declare that:

1. I am a U.S. citizen residing at 6353 Westchester Street, Houston, Texas. I am Associate Professor of Surgery and Deputy Chairman of the Department of Head and Neck Surgery at the University of Texas M.D. Anderson Cancer Center. A copy of my curriculum vitae outlining my education and research training is attached (Exhibit A).

2. I am the inventor of the above-captioned application and a portion of my research has been sponsored by Introgen Therapeutics, Inc., a company that has licensed this technology.

3. I am a co-author of Clayman *et al.*, published in *Cancer Research* (Exhibit B), along with Drs. Adel K. El-Naggar, Jack A. Roth, Wei-Wei Zhang, Helmuth Goepfert, Dorothy L. Taylor, and Ta-Jen Liu. I also am co-author of Liu *et al.*, published in *Cancer Research* (Exhibit C), along with Drs. Liu, El-Naggar, Taylor, Timothy J. McDonnell, Kim D. Steck, and Mary Wang.

4. Drs. El-Naggar, Roth, Zhang, Goepfert, Taylor, Liu, McDonnell, Steck and Wang, the non-inventor co-authors of this paper, did not contribute to the conception of the present invention of using Adp53 for the treatment of head and neck cancer. Each of these individuals acted under the supervision and direction of myself in generating the results reported in these papers, or as a reviewer of the manuscripts prior to publication.

5. Dr. Adel K. El-Naggar performed the pathologic analysis in Exhibit B and the fluorescent analysis in Exhibit C.

6. Dr. Jack A. Roth provided the Adp53 vector in Exhibits B and C.

7. Dr. Wei-Wei Zhang developed the Adp53 vector in Exhibit B.

8. Dr. Helmuth Goepfert reviewed the Exhibit B manuscript prior to publication.

9. Dr. Dorothy L. Taylor developed and maintained the head and neck cancer cell lines, and assisted in the experiments described in Exhibits B and C.

10. Dr. Ta-Jen Liu performed the *in vitro* propagation of the Adp53 vector in Exhibit B and performed the DNA fragmentation analysis in Exhibit C.

11. Dr. Timothy J. McDonnell reviewed the Exhibit C manuscript prior to publication.

12. Dr. Kim D. Steck performed the technical fluorescent sorting studies in Exhibit C.

13. Dr. Mary Wang performed infection assays and viral propagation in Exhibit C.

14. I hereby declare that all statements made herein of my knowledge are true and that all statements made herein on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the U.S. Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

6/18/98
Date

Gary L. Clayman, D.D.S., M.D.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
GARY L. CLAYMAN

Serial No.: 08/758,033

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DECLARATION UNDER 37 C.F.R. §1.131 OF DR. GARY CLAYMAN

BOX AF
Assistant Commissioner for Patents
Washington, D.C. 20231

I, Gary Clayman, declare that:

1. I am a U.S. citizen residing at 6353 Westchester St., Houston, Texas. I am Associate Professor of Surgery and Deputy Chairman of the Department of Head and Neck Surgery at the University of Texas M.D. Anderson Cancer Center.

2. I am the Gary Clayman named as an inventor of the above-captioned application. A portion of my research has been sponsored by Introgen Therapeutics, Inc., the exclusive licensee of this application.

3. In January of 1995, I published a paper (Liu *et al.*, *Cancer Res.* 55:1-6 (1995)) that reported the Ad-p53 infection of cell lines with both mutated and wild-type p53. A similar report was in July of the same year (Clayman *et al.*, *Cancer Res.* 55:3117-3122 (1995)). I understand that the Examiner in charge of examining the referenced application has previously taken the position that these papers teach the use of adeno-p53 in the therapy of tumors *in vivo*, including the therapy of p53-positive tumors (Office Action of 2/17/99).

4. The studies set forth in these papers demonstrate that I had achieved the subject matter that they disclose in the United States at least as of their date of publication, the earliest publication date as between the two being January, 1995).

5. I understand that the papers of Katayose *et al.*, *Clin. Cancer Res.* 1:889-897 (1995) and Srivastava *et al.*, *Urology* 46:843-848 (1995), both published after January, 1995, are being cited against various claims of the present application for their alleged teaching of *in vitro* studies using a p53-expressing adenovirus to infect tumor cells. Based on the earlier publication of my two articles referenced above, it is clear that I had in my possession at least equivalent, and indeed more extensive, data than is taught in the Katayose and Srivastava references at a time prior to their respective publication dates.

6. I declare that all statements made herein of myr own knowledge are true, and that all statements of my own belief are believed to be true, and further that these statements were made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this patent, and any reexamination certificate issuing thereon.

11/8/99
Date

Dr. Gary Clayman

(c)

D.2

; E. Johnson, in preparation).
23. In reviewing unpublished studies from my laboratory (T. E. Johnson, unpublished data) involving smaller populations (no larger than 50 worms) and only thrice-weekly survival assessments, 14 of 19 comparisons show lower mutual mortality rates for age-1 whereas only 4 of 19 show similar changes for the exponential Gompertz component. It thus seems that larger populations and more frequent assays are required to detect the effects described in this report.
24. D. E. Harrison, *Growth Dev. Aging* 52, 207 (1988); T. E. Johnson, *ibid.*, p. 207; M. R. Rose, *ibid.*, p. 209; B. Charlesworth, *ibid.*, p. 211.
25. J. F. Fries, *N. Engl. J. Med.* 303, 130 (1980).
26. D. B. Brock, J. M. Guralnick, J. A. Brody, in *Handbook of the Biology of Aging*, E. L. Schneider and J. W. Rowe, Eds. (Academic Press, New York, ed. 3, 1990), pp. 8–23.
27. E. L. Schneider and J. A. Brody, *N. Engl. J. Med.* 309, 854 (1983).
28. I thank B. Conley, D. Friedman, and P. Fitzpatrick for careful data collection; A. Brooks, C. Finch, D. Lindsay, D. Fulker, G. Stein, and M. Witten for helpful discussions; and R. Miles for editorial assistance. This work was supported by NIH grants R01 AG05720, R01 AG08332, and K04 AG00869

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Suppression of Human Colorectal Carcinoma Cell Growth by Wild-Type p53

SUZANNE J. BAKER, SANFORD MARKOWITZ, ERIC R. FEARON, JAMES K. V. WILLSON, BERT VOGELSTEIN*

Mutations of the p53 gene occur commonly in colorectal carcinomas and the wild-type p53 allele is often concomitantly deleted. These findings suggest that the wild-type gene may act as a suppressor of colorectal carcinoma cell growth. To test this hypothesis, wild-type or mutant human p53 genes were transfected into human colorectal carcinoma cell lines. Cells transfected with the wild-type gene formed colonies five- to tenfold less efficiently than those transfected with a mutant p53 gene. In those colonies that did form after wild-type gene transfection, the p53 sequences were found to be deleted or rearranged, or both, and no exogenous p53 messenger RNA expression was observed. In contrast, transfection with the wild-type gene had no apparent effect on the growth of epithelial cells derived from a benign colorectal tumor that had only wild-type p53 alleles. Immunocytochemical techniques demonstrated that carcinoma cells expressing the wild-type gene did not progress through the cell cycle, as evidenced by their failure to incorporate thymidine into DNA. These studies show that the wild-type gene can specifically suppress the growth of human colorectal carcinoma cells in vitro and that an in vivo–derived mutation resulting in a single conservative amino acid substitution in the p53 gene product abrogates this suppressive ability.

ONE COPY OF THE SHORT ARM OF chromosome 17, which harbors the p53 gene, is lost in many human tumors, including those of the colon and rectum (*1–3*). In the majority of human colon carcinomas with allelic deletions of chromosome 17p, the remaining p53 allele contains a missense mutation (*3, 4*). In addition to colorectal carcinomas, p53 gene mutations have also been found in conjunction with chromosome 17p allelic deletions in tumors of the brain, breast, lung, and bone (*4–6*). These studies are consistent with the hypothesis that the normal (wild-type) p53 gene product may function as a suppressor of neoplastic growth, and that mutation or deletion, or both, of the wild-type gene inactivates this suppression. This hypothesis has been supported by studies in rodent cells. For example, p53 alleles are often rearranged or mutated as a result of viral integration events in Friend virus–induced mouse erythroleukemias (*7*). Additionally, in transfection studies, the wild-type murine p53 gene has been shown to inhibit the transforming ability of mutant p53 genes in rat embryo fibroblasts (*8*). Other studies, however, have suggested that expression of the wild-type p53 gene product is necessary (not inhibitory) for cell growth (*9, 10*). Thus, the effect of wild-type and mutant p53 genes on cell growth may depend on the cell type examined. We now show that expression of the wild-type p53 gene in human colorectal carcinoma cells dramatically inhibits their growth. Moreover, a p53 gene mutant cloned from a human colorectal carcinoma was biologically inactive in this respect, as it was incapable of inhibiting such growth.

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Table 1. Colony formation after transfection with wild-type and mutant p53 expression vectors. For each experiment, one or two 75-cm² flasks were transfected (*13*), and the total colonies counted after 3 to 4 weeks of selection in geneticin (0.8 mg/ml). Exp., experiment.

Cell line	Exp.	No. of geneticin-resistant colonies formed	
		pC53-SCX3 (mutant)	pC53-SN3 (wild-type)
SW837	1	754	66
	2	817	62
SW480	1	449	79
	2	364	26
RKO	1	1858	190
	2	1825	166
VACO 235	1	18	16
	2	26	28

The colorectal carcinoma lines SW480 and SW837, which are representative of 75% of colon carcinomas, have each lost one copy of chromosome 17p (including the p53 gene), and the remaining p53 allele is mutated (*3, 4*). The SW837 line contains an arginine to tryptophan mutation at codon 248 (*4*). The SW480 line contains two point mutations, arginine to histidine at codon 273 and proline to serine at codon 309 (*4*). The substitutions at codon 248 and 273 are typical of those observed in human tumors, occurring within two of the four mutation "hot spots" (*4*). For the transfection studies, we constructed a vector, pCMV-Neo-Bam, engineered to contain two independent transcription units (*11*). The first unit comprised a cytomegalovirus (CMV) promoter/enhancer upstream of a site for insertion of the cDNA sequences to be expressed, and splice and polyadenylation sites to ensure appropriate processing. The second transcription unit included a herpes simplex virus (HSV) thymidine kinase promoter/enhancer upstream of the neomycin resistance gene, allowing for selection of transfected cells in geneticin (*11*). A wild-type p53 cDNA was inserted into pCMV-Neo-Bam to produce pC53-SN3. Similarly, a vector, pC53-SCX3, expressing a mutant cDNA from human colorectal tumor CX3, was also constructed. The only difference between pC53-SN3 and pC53-SCX3 was a single nucleotide (C to T) resulting in a substitution of alanine for valine at p53 codon 143 in pC53-SCX3 (*12*).

The constructs were transfected into SW837 and SW480 cells (*13*), and geneticin-resistant colonies were counted 3 weeks later. Cells transfected with pC53-SN3 formed five- to tenfold fewer colonies than those transfected with pC53-SCX3 in both recipient cell types (Table 1). In both

SW837 and SW480 cells, the number of colonies produced by the expression vector pCMV-Neo-Bam (without a p53 cDNA insert) was similar to that induced by the pC53-SCX3 construct.

These results suggested that the wild-type p53 gene inhibited the clonal growth of both the SW837 and SW480 cell lines; however, a significant number of colonies formed after transfection of the wild-type construct. If wild-type p53 expression were truly inhibitory to cell growth, one would expect that no colonies would form or that p53 expression in the colonies that did form would be reduced compared to that produced with the mutant p53 cDNA construct. To evaluate this issue, we expanded independent SW480 and SW837 colonies into lines, and ribonuclease (RNase) protection analysis was performed to determine the amount of p53 mRNA expressed from the exogenously introduced sequences. Twelve of 31 lines (38%) derived from transfection with the pC53-SCX3 construct were found to express the exogenous mutant p53 mRNA. This percentage was consistent with results expected in human cells transfected with a vector containing two independent transcription units (*14*). In contrast, no expression of exogenous p53 wild-type mRNA was seen in any of 21 clonal lines established from either SW480 or SW837 cells transfected with the pC53-SN3 vector (Fig. 1A). These RNase protection results were supported by analysis of the exogenous p53 DNA sequences within the clones. All of the p53-expressing clones derived from the pC53-SCX3 transfection contained an intact copy of the exogenous p53 gene (Fig. 1B). In contrast, in all the clones derived from the pC53-SN3 transfection, the exogenous p53 sequences were deleted or rearranged (Fig. 1B).

The results from individual clones were further supported by the analysis of pooled clones, in which numerous colonies could be simultaneously assessed. Forty or more clones from two to three separate transfection experiments were pooled and analyzed approximately 3 weeks after transfection. RNase protection studies showed substantial expression of exogenous mutant sequences in the pooled clones, whereas expression of wild-type sequences was not detectable (Fig. 2A). Results from Southern (DNA) blotting were consistent with the RNase protection studies, in that pooled colonies from the wild-type transfectants had no detectable unrearranged exogenous p53 sequences, in contrast to the intact p53 sequences in colonies derived from the mutant p53 cDNA expression vector (Fig. 2B).

The conclusions made from the above experiments are dependent on the assumption that p53 protein was produced in the transfected cell lines. Clones containing exogenous mutant p53 sequences produced p53 mRNA at a concentration 1.5 to 3.5 times higher than that produced by the endogenous p53 gene (Figs. 1A and 2A). Immunoblot analysis showed that there was a concomitant small increase in p53 protein expression in the transfectants (1.5- to 3-fold) compared to the untransfected cells (*15*). However, this increase was difficult to measure quantitatively, since these cells produced significant amounts of endogenous p53 protein that (unlike endogenous p53 mRNA) could not be distinguished from that produced by the vectors. To confirm that transfected human cells expressed p53 protein from our constructs, we studied an additional colorectal carcinoma cell line (RKO). Although RKO cells did not contain a mutation within the susceptible p53 coding sequences (*16*), they expressed low concentrations of p53 mRNA compared to normal colorectal mucosa or the other lines studied and did not produce detectable amounts of protein (*15*).

Results of colony formation assays in transfected RKO cells were similar to those in SW480 and SW837 cells. Colony formation by wild-type p53 gene transfectants occurred with a tenfold decrease in efficiency compared to the mutant p53 construct (Table 1). Immunocytochemical detection of p53 protein in transfected RKO cells (*17*) revealed that approximately equal numbers of cells expressed wild-type and mutant protein 6 hours after transfection. A twofold difference was found at 24 hours, and this

Table 2. Immunocytochemistry and [^{3}H]thymidine incorporation of transfected RKO cells. To determine p53 expression, we split RKO cells into eight flasks and individually transfected them with either pC53-SCX3 or pC53-SN3 (*13*). At the indicated times after transfection, cells from each flask were fixed and stained with a monoclonal antibody to p53 protein (*17*). At least 1500 cells were counted for each determination. To determine [^{3}H]thymidine incorporation, we split RKO cells into duplicate flasks and individually transfected them with either pC53-SCX3 or pC53-SN3 (*13*). Forty-six hours after transfection, the cells were incubated with [^{3}H]thymidine for 2 hours, then fixed and stained with a monoclonal antibody to p53 protein (*17*). Evaluation of thymidine incorporation in the transfected cells was performed as previously described (*19*). At least 50 p53-expressing cells and 400 p53-nonexpressing cells (determined immunocytochemically with antibody to p53) were assessed for each determination of [^{3}H]thymidine uptake.

Plasmid	Percent of cells expressing p53 protein at				Percent of cells incorporating [^{3}H]thymidine in	
	6 hours	24 hours	48 hours	96 hours	p53 expressors	p53 nonexpressors
pC53-SCX3	2.0	11	4.3	2.0	24	31
pC53-SN3	1.9	5.2	0.3	0.2	1.7	33

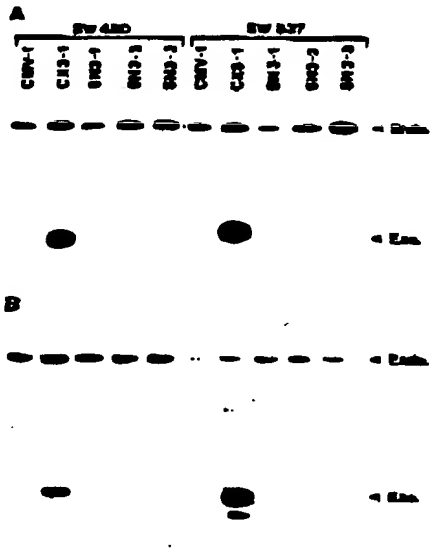

Fig. 1. (**A**) RNase protection analysis of transfected clonal lines. A labeled antisense p53 probe, which distinguishes between endogenous p53 mRNA and exogenous p53 mRNA, was hybridized with total cellular RNA from representative lines established from independent geneticin-resistant clones (*23*). After digestion with RNase A, the resulting hybridization products were separated by electrophoresis on denaturing polyacrylamide gels and autoradiographed (*24*). The labeled probe comprised nucleotides 1450 to 1788 relative to the p53 translation initiation site. Endogenous p53 mRNA included all of the p53 sequences represented in the labeled probe, so a 388-bp hybridization product (Endo.) was protected from RNase digestion. The exogenous p53 mRNA produced from the expression vector, however, only extended to nucleotide 1671; hybridization to exogenous p53 mRNA followed by RNase digestion therefore gave rise to a 221-bp fragment (Exo.). Clonal lines designated CMV, CX3, and SN3 were transfected with pCMV-Neo-Bam, pC53-SCX3, and pC53-SN3, respectively. (**B**) Southern blot analysis of transfected clonal lines. DNA from representative clonal lines (Fig. 1A) was digested with Bam HI, separated by electrophoresis on an agarose gel, transferred to nylon, and hybridized to a labeled p53 gene probe (*25*). The exogenous p53 gene from the expression vector was present in a 1.8-kb Bam HI fragment (Exo.) if it had not been rearranged in the cell. The endogenous p53 gene (Endo.) gave rise to a 7.8-kb Bam HI fragment.

difference increased with time (Table 2). These observations are consistent with the greater stability of mutant compared to wild-type p53 protein noted previously (*18*). However, transient mRNA expression was also significantly lower in the SN3 transfectants compared to the SCX3 transfectants at 48 and 96 hours (*15*), supporting the idea that RKO cells expressing wild-type p53 were at a selective disadvantage compared to those producing mutant p53 products.

To obtain additional evidence that cells expressing p53 were inhibited in their growth potential, we examined the effect of p53 gene expression on DNA synthesis in transfected RKO cells. Forty-eight hours after transfection, RKO cells were labeled with [^{3}H]thymidine for 2 hours. The cells were subsequently fixed, immunocytochemically stained for the presence of p53 protein, and autoradiographed (*19*). The number of cells undergoing DNA replication was only slightly lower in cells producing exogenous mutant p53 protein than in cells that did not express any detectable p53 protein. Expression of the wild-type protein, however, dramatically inhibited the incorporation of thymidine (Table 2).

These results all suggested that wild-type p53 exerted an inhibitory effect on the growth of carcinoma cells in vitro. To evaluate whether this inhibitory effect was cell type–specific, we transfected colorectal epithelial cells derived from a benign tumor of the colon (the VACO 235 adenoma cell line). Previous studies have shown that most adenomas contain two copies of chromosome 17p and express wild-type p53 mRNA at concentrations similar to that of normal colonic mucosa (*1*, *15*). Analogously, the p53 alleles of the VACO 235 cell line were sequenced and found to be wild type (*16*), and the expression of p53 mRNA was found to be similar to that of normal colorectal mucosa (*15*). In contrast to the results seen with SW480, SW837, and RKO cells, the pC53-SN3 and pC53-SCX3 constructs produced similar numbers of geneticin-resistant colonies after transfection of the VACO 235 line (Table 1). We considered, however, that the most definitive test for differential growth inhibition by wild-type versus mutant p53 genes involved analysis of exogenous p53 expression in pooled transfectants. Through such analysis, a large number of colonies could be examined simultaneously and the expression of exogenous mutant and wild-type p53 genes directly compared. Striking differences in the relative expression from the transfected genes were seen in all three carcinoma cell lines tested. VACO 235 transfectants, however, expressed similar amounts of exogenous p53 mRNA from either pC53-SN3 (wild-type) or pC53-SCX3 (mutant) p53 constructs (Fig. 2A).

In summary, our results suggest that expression of the wild-type p53 gene in colorectal carcinoma cell lines was incompatible with proliferation. The inhibitory effects of wild-type p53 were specific in two ways. First, a single point mutation in a p53 gene construct abrogated its suppressive properties as measured by three separate assays (colony formation, exogenous p53 expression in transfected clones, and thymidine incorporation). The CX3 mutant provided a control for gene specificity, as it contained only one conservative mutation, resulting in a substitution of one hydrophobic amino acid (alanine) for another (valine) at a single codon. Second, the growth-suppressive effect of the wild-type p53 construct was cell type–specific. Introduction of the wild-type vector into the VACO 235 adenoma cell line had no measurable inhibitory effect compared to the mutant p53 vector. There are several differences between the cell lines that could account for the differential effect of the introduced vectors. Regardless of the basis for the difference, the results with the VACO 235 cell line minimize the possibility that the wild-type p53 construct had some nonspecific, toxic effect on recipient cells; the effect was cell type–dependent.

The transfection and expression results of Table 1 and Fig. 2A suggest that cells at the premalignant stages of tumor progression (VACO 235) may be less sensitive to the inhibitory effects of wild-type p53 than malignant cells (SW480, SW837, and RKO). This hypothesis is consistent with previous results that suggest the wild-type p53 is less inhibitory to the growth of normal rat embryo fibroblasts than to their oncogene-transfected derivatives (*8*). This sensitivity may only be relative; expression of the wild-type gene at high concentrations might inhibit the growth of any cell type, including non-neoplastic cells, by overwhelming normal regulatory processes such as phosphorylation (*20*, *21*). Genetic alterations that occur during the progression of colorectal tumors (*22*) may increase the sensitivity of cells to p53 inhibition, making wild-type p53 expression a key, rate-limiting factor for further tumor growth and expansion. At this point, and not before, mutations in the p53 gene would confer a selective growth advantage to cells in vivo, which would explain the frequent occurrence of p53 gene mutations and allelic loss only in the more advanced stages of colorectal tumorigenesis (*1*, *22*).

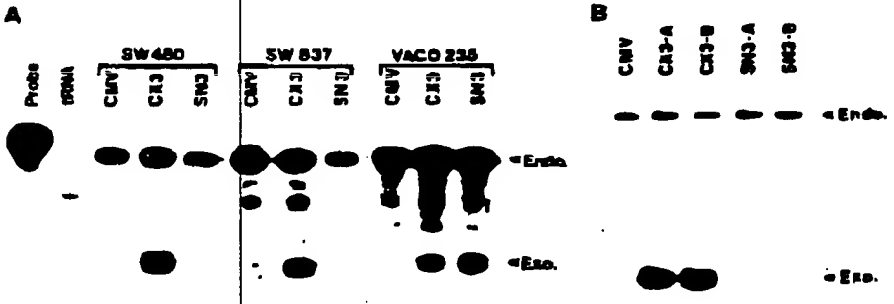

Fig. 2. (**A**) Expression analysis of pooled clones. One to four flasks containing a total of at least 40 independent geneticin-resistant clones transfected with pC53-SCX3 or pC53-SN3 (designated CX3 and SN3, respectively) were pooled for RNA preparation (*23*). Geneticin-resistant clones formed after transfection of pCMV-Neo-Bam, a vector devoid of p53 sequence (designated CMV), were used as a negative control. RNase protection was performed as described in Fig. 1A. Endogenous and exogenous p53 mRNA are designated as Endo. and Exo., respectively (see legend to Fig. 1A). (**B**) Southern blot analysis of SW480 pooled clones. DNA from pooled clones of SW480 cells was digested with Bam HI, separated by agarose gel electrophoresis, transferred to nylon, and hybridized with a labeled probe from the p53 gene as described (*23*). The lanes represent pooled clones from SW480 cells transfected with the following: lane CMV, pCMV-Neo-Bam; lanes CX3-A and CX3-B, pC53-SCX3 (two independent pools); and lanes SN3-A and SN3-B, pC53-SN3 (two independent pools). The 7.8-kb fragment from the endogenous p53 gene is indicated as Endo., and the 1.8-kb fragment from the exogenously introduced DNA is indicated as Exo.

REFERENCES AND NOTES

1. E. R. Fearon, S. R. Hamilton, B. Vogelstein, *Science* 238, 193 (1987).
2. J. Yokota *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 84, 9252 (1987); C. D. James *et al.*, *Cancer Res.* 48, 5546 (1988); J. Toguchida *et al.*, *ibid.*, p. 3939; P. Delattre *et al.*, *Lancet* ii, 353 (1989); J. Mackay *et al.*, *ibid.*, p. 1384; P. Devilee *et al.*, *Genomics* 5, 554 (1989); Y. C. Tsai, P. W. Nichols, A. L. Hiti, D. G. Skinner, P. A. Jones, *Cancer Res.* 50, 44 (1990).
3. S. J. Baker *et al.*, *Science* 244, 217 (1989).
4. J. M. Nigro *et al.*, *Nature* 342, 705 (1989).
5. T. Takahashi *et al.*, *Science* 246, 491 (1989); R. Iggo *et al.*, *Lancet* 335, 675 (1990).
6. D. Wolf and V. Rotter, *Proc. Natl. Acad. Sci. U.S.A.* 82, 790 (1985); H. Mumata, C. Miller, H. P. Koeffler, H. Battifora, M. J. Clare, *ibid.* 84, 7716 (1987); J. Romano *et al.*, *Oncogene* 4, 1483 (1989); R. Mashal *et al.*, *Blood* 76, 180 (1990).

... and M. Mowat, *J. Virol.* 62, 4752 (1988); D. G. Munroe, R. Rowinsky, A. Bernstein, *Oncogene* 2, 621 (1988).
6. C. A. Finlay, P. H. Hinds, A. J. Levine, *Cell* 57, 1083 (1989); D. Ginsahu, D. Michaelovitz, S. Ginsahu, O. Pinhasi-Kimhi, M. Oren, *Proc. Natl. Acad. Sci. U.S.A.* 86, 8763 (1989).
7. W. E. Mercer, C. Avignolo, R. Baserga, *Mol. Cell Biol.* 4, 276 (1984).
8. O. Shohat, M. Greenberg, D. Reisman, M. Oren, V. Rotter, *Oncogene* 1, 277 (1987).
11. The expression vector pCMV-Neo-Bam was derived from plasmid BCMGNeo-mIL2 [H. Karasuyama, N. Tohyama, T. Tada, *J. Exp. Med.* 169, 13 (1989)] by excision of the human beta globin sequences and bovine papilloma virus sequences with Bam HI and Not I. Next, the interleukin 2 (IL-2) sequences present at the unique Xho I site were removed, and the Xho I site was changed to a Bam HI site by linker addition. The vector included CMV promoter/enhancer sequences, which could drive expression of the insert at the Bam HI site, and splicing and polyadenylation sites derived from the rabbit beta globin gene, which ensured proper processing of the transcribed insert in the cells. A pBR322 origin of replication and beta-lactamase gene facilitated growth of the plasmid in *Escherichia coli*. The plasmid conferred geneticin resistance through expression of the neomycin resistance gene under separate control of an HSV thymidine kinase promoter.
12. A 1.8-kb Xba I fragment, extending from nucleotide −130 to 1671 relative to the translation initiation site, was isolated from wild-type or CX3 cDNA clones (3). The fragment was blunt-ended with the Klenow fragment of DNA polymerase, ligated to Bam HI linkers, and cloned into the unique Bam HI site in the expression vector pCMV-Neo-Bam.
13. SW480 and SW837 were obtained from American Type Culture Collection (ATCC). RKO cells were obtained through the generosity of M. Brattain. VACO 235 cells are described by J. K. V. Willson *et al.* [*Cancer Res.* 47, 2704 (1987)]. For transfection, carcinoma cells at 30 to 60% confluence were incubated in a 75-cm² flask in 6 ml of Opti-mem (Gibco) with 5 μg of plasmid DNA and 30 μg of lipofectin [P. L. Felgner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 84, 7413 (1987)]. After 5 to 16 hours, the Opti-mem was replaced with Dulbecco's or McCoy's 5A medium containing 10% fetal calf serum. Selection in geneticin (0.8 mg/ml) began 36 to 48 hours after transfection for colony formation assays. Electroporation was used to transfect VACO 235 cells essentially as described by H. Potter, L. Weir, and P. Leder [*Proc. Natl. Acad. Sci. U.S.A.* 81, 7161 (1984)].
14. Previous studies have shown that, in contrast to rodent cells, primate cells are able to integrate only a small amount of foreign DNA (approximately 6 kb), so that only 10 to 30% of clones selected for the expression of one transcription unit also contain the second unit in an intact form [F. Collabere-Garapin, M. Rhyner, I. Stephany, P. Kourilsky, A. Garapin, *Gene* 50, 279 (1986); J. H. J. Hoeijmakers, H. Odijk, A. Westerveld, *Exp. Cell Res.* 169, 111 (1987); L. Mayne *et al.*, *Gene* 66, 65 (1988); S. W. Dean, L. Kunda, H. R. Sykes, A. R. Lehmann, I. A. Wise, *Exp. Cell Res.* 183, 473 (1989)].
15. S. J. Baker, A. C. Preisinger, B. Vogelstein, unpublished data.
16. The p53 gene sequences in exons 5, 6, 7, 8, and 9 were examined essentially as described in (4). All previously noted point mutations in p53 genes have involved one of these exons (see 3–5).
17. Approximately 5×10^5 cells were cytocentrifuged onto polylysine-coated slides, fixed for 10 min in formalin, and permeabilized for 5 min in 0.5% Triton X-100. A mouse monoclonal antibody against human p53 protein (Ab1801) in combination with the ABC immunoperoxidase system (Vector Laboratories), was used for immunocytochemical detection of p53 protein [L. Banks, G. Matlashewski, L. Crawford, *Eur. J. Biochem.* 159, 529 (1986)]. Ten to 20 randomly selected microscopic fields were analyzed per slide.
18. C. A. Finlay *et al.*, *Mol. Cell. Biol.* 8, 531 (1988); P. W. Hinds *et al.*, in preparation.
19. Cells were grown for 2 hours in McCoy's 5A medium with 10% fetal calf serum and [^{3}H]thymidine (10 μCi/ml of 50 Ci/mmol, New England Nuclear). After immunocytochemical staining (17), slides were dehydrated in ethanol, dipped in NTB-2 emulsion (Kodak), and exposed for 2 weeks at 4°C. Autoradiographs were developed for 2 min in D-19 and stabilized in Rapid Fix (Kodak).
20. A. Samad, C. W. Anderson, R. B. Carroll, *Proc. Natl. Acad. Sci. U.S.A.* 83, 8872 (1986); D. W. Meek and W. Eckhart, *Mol. Cell. Biol.* 8, 461 (1988); J. Bischoff *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
21. L. Diller, S. J. Baker, B. Vogelstein, S. Friend, unpublished data.
22. B. Vogelstein *et al.*, *N. Engl. J. Med.* 319, 525 (1988); B. Vogelstein *et al.*, *Science* 244, 207 (1989).
23. Total cellular RNA was isolated by the acid guanidium extraction method [P. Chomczynski and N. Sacchi, *Anal. Biochem.* 162, 156 (1987)].
24. RNA (15 μg) from each sample was used in RNase protection experiments. A ^{32}P-labeled RNA probe comprising nucleotides 1450 to 1788 relative to the p53 translation initiation site was generated in vitro from a p53 cDNA subclone in Bluescript with T7 polymerase. Ribonuclease protection was performed as previously described [E. Winter, F. Yamamoto, C. Almoguera, M. Perucho, *Proc. Natl. Acad. Sci. U.S.A.* 82, 7575 (1985); R. M. Myers, Z. Lann, T. Maniatis, *Science* 230, 1242 (1985)]. Autoradiographs were exposed for 16 to 20 hours.
25. DNA purification, restriction endonuclease digestion, electrophoresis, transfer, and hybridization were performed as described (1, 9). The hybridization probe was a 1.8-kb Xba I fragment of p53 cDNA (12).
26. We thank A. Preisinger, K. Molkentin, and J. Jackson for technical assistance. This work was supported by grants GM 07184, GM 07309, CA 43703, CA 45967, CA 43460, CA 51504, and CA 35494 from the NIH.

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A β_3 Integrin Mutation Abolishes Ligand Binding and Alters Divalent Cation–Dependent Conformation

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The ligand-binding function of integrin adhesion receptors depends on divalent cations. A mutant $\alpha_{IIb}\beta_3$ integrin (platelet gpIIb/IIIa) that lacks ligand recognition shows immunologic evidence of a perturbed interaction with divalent cations. This was found to be caused by a G → T mutation that resulted in an $Asp^{119} \rightarrow Tyr^{119}$ substitution in the β_3 subunit. This residue is proximal to bound ligand and is in a conserved region among integrins that are enriched in oxygenated residues. The spacing of these residues aligns with the calcium-binding residues in EF hand proteins, suggesting interaction with receptor-bound divalent cation as a mechanism of ligand binding common to all integrins.

CELL-CELL AND CELL-MATRIX ADHESIVE interactions are essential to development, inflammation, hemostasis, and immune recognition. The integrins are a broadly distributed family of structurally related receptors that contribute to these adhesive reactions by recognition of a multiplicity of extracellular matrix protein ligands including laminin, collagens, fibrinogen, and bone sialoprotein (*1*). In addition, integrins participate in cell-cell interactions by recognition of integral membrane protein ligands including the intercellular adhesion molecules ICAM-1 and ICAM-2 and vascular cell adhesion molecule–1 (VCAM-1) (*2*). Although the integrins differ in ligand recognition specificity, a requirement for millimolar concentrations of physiologic divalent cations is common to the primary recognition function of all integrins (*3*). This dependence of function on divalent cations can be attributed to a low-affinity divalent cation–binding site within the integrins, because millimolar Ca^{2+} or Mg^{2+} can modulate the conformation of a prototype integrin, platelet membrane glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$, also known as gpIIb/IIIa), which is detectable by a monoclonal antibody (MAb) PMI-1 (*4*). Loss of the epitope recognized by this MAb directly correlates with the capacity of $\alpha_{IIb}\beta_3$ to bind fibrinogen. The Cam variant of Glanzmann's thrombasthenia (*4*) is an autosomal recessive hereditary disorder of $\alpha_{IIb}\beta_3$ that is associated with the inability of this integrin to recognize macromolecular (*4*) or synthetic peptide (*5*) ligands. In addition, divalent cations do not regulate the expression of the PMI-1 epitope in Cam platelets (*4*). These characteristics indicate that the presumptive mutation in the Cam receptor leads to defects in binding of both divalent cations and primary ligands. To elucidate the structural basis of integrin function, we identified the point mutation in $\alpha_{IIb}\beta_3$ that causes the Cam variant of Glanzmann's thrombasthenia.

Total RNA was isolated from platelets of normal donors and two affected siblings with Cam variant. For initial sequencing, we

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Growth suppression of human breast cancer cells by the introduction of a wild-type p53 gene

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Mutations in the p53 gene are associated with a wide variety of human tumors, including those of the breast. To assess functionally the role of the p53 gene in the development of human breast cancer, we introduced either wild-type or mutant p53 cDNA into three human breast cancer cell lines by DNA transfection. The cell lines MDA-MB 468 and T47 D contain only single mutated copies of the p53 gene, whereas the status of p53 in the breast cancer cell line MCF 7 remains equivocal. Following transfection, MCF 7 cells continued to grow unaffected both *in vitro* and *in vivo* in the presence of high levels of expression of the exogenous wild-type p53 gene. In contrast, however, the continued expression of an exogenous wild-type p53 gene was incompatible with cellular growth in both the MDA-MB 468 and T47 D cell lines. Elevated levels of expression of the exogenous mutant p53 gene did not alter the growth of the cell lines *in vitro*. These data strongly suggest that the wild-type p53 gene can function as a suppressor of cellular growth in breast cancer cells. That the wild-type p53 gene does not suppress the growth of MCF 7 cells indicates that at least some human breast tumors can arise without functional inactivation of the p53 gene by mutation. These tumors may represent a separate prognostic group.

Introduction

The p53 gene is a nuclear phosphoprotein (Lane & Crawford, 1979) which has been implicated in the normal proliferation and neoplastic transformation of cells (Lane & Benchimol, 1990). It is expressed at low levels in non-transformed cells but is often elevated in tumor-derived or transformed cell lines (Lane & Benchimol, 1990). Several studies support the hypothesis that the p53 gene can function as a dominant transforming oncogene (Eliyahu *et al.*, 1984; Jenkins *et al.*, 1984; Parada *et al.*, 1984). Recently, however, it has been demonstrated that these studies employed mutated p53 genes (Lane & Benchimol, 1990), and that the wild-type gene is incapable of transformation (Eliyahu *et al.*, 1988; Finlay *et al.*, 1988; Hinds *et al.*, 1989). Indeed, the wild-type gene can even inhibit the activity of transforming genes in transfection assays (Finlay *et al.*, 1989). These data would therefore suggest that the wild-type p53 gene can act as a suppressor of cellular growth.

There is strong evidence implicating mutations in the p53 gene in the etiology of many human cancers (Baker *et al.*, 1989; 1990; Nigro *et al.*, 1989; Takahashi *et al.*, 1989; Diller *et al.*, 1990; Iggo *et al.*, 1990; Mercer *et al.*, 1990; Mulligan *et al.*, 1990; Rodrigues *et al.*, 1990). Evidence has recently been obtained for the functional suppression of the cellular growth of several different human cancer cell lines, including colon cancer (Baker *et al.*, 1990), glioblastoma (Mercer *et al.*, 1990) and osteosarcoma (Chen *et al.*, 1990; Diller *et al.*, 1990), following DNA transfection or retroviral transfer of the wild-type p53 gene.

Evidence is accumulating that mutations in the p53 gene are important in the development of human breast cancer (Nigro *et al.*, 1989; Bartek *et al.*, 1990a). Allele losses have frequently been observed on the short arm of chromosome 17 in human breast tumors (Mackay *et al.*, 1988; Devilee *et al.*, 1989; Varley *et al.*, 1991), consistent with there being a tumor-suppressor gene(s) in this region. Although there may be two regions of allele loss on chromosome 17p (Coles *et al.*, 1990), one is known to include the p53 gene (Coles *et al.*, 1990; Devilee *et al.*, 1990) at 17p13 (Isobe *et al.*, 1986; McBride *et al.*, 1986). Frequent overexpression of the p53 gene has been reported in breast tumors (Crawford *et al.*, 1984), and there is a high correlation between elevated expression of the p53 gene and loss of heterozygosity on the short arm of chromosome 17 (Thompson *et al.*, 1990). Point mutations in the p53 gene have been detected in both breast cancer cell lines and primary tumors (Nigro *et al.*, 1989; Bartek *et al.*, 1990a; 1990b; Prosser *et al.*, 1990; Varley *et al.*, 1991), and abnormal histochemical staining using anti-p53 antibodies has been reported in approximately 50% of breast tumors examined (Cattoretti *et al.*, 1988; Bartek *et al.*, 1990a). Interestingly, mutations of the p53 gene correlate strongly with abnormal histochemical staining (Bartek *et al.*, 1990b; Iggo *et al.*, 1990; Rodrigues *et al.*, 1990). Recently, two groups (Malkin *et al.*, 1990; Srivastava *et al.*, 1990) have shown germ line p53 gene mutations in fibroblasts derived from both affected and non-symptomatic individuals exhibiting the hereditary cancer disease Li–Fraumeni syndrome. Patients with this syndrome can develop a variety of soft-tissue cancers, and often develop breast cancer at an early age (Li & Fraumeni, 1969). Taken together, these results strongly suggest that the wild-type p53 gene may function as a suppressor of cellular growth in human breast cancer cells.

To obtain functional evidence for this hypothesis we have introduced both wild-type and mutant p53 cDNAs by DNA transfection into the breast cancer cell lines MDA-MB 468, T47 D and MCF 7. MDA-MB 468 and T47 D have previously been shown to contain only a

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single, mutated copy of the p53 gene (Nigro *et al.*, 1989; Bartek *et al.*, 1990b). The status of p53 in the MCF 7 cell line remains to be determined.

We present data which show that the wild-type p53 gene functions as a suppressor of cellular growth in the cell lines MDA-MB 468 and T47 D but has little effect upon MCF 7 cells. The data presented support a role for the wild-type p53 gene in the suppression of cellular growth in some, but not all, breast cancer cells.

Results

Status of the p53 gene in breast cancer cell lines

It has been shown previously that the cell lines MDA-MB 468 and T47 D are hemizygous for the p53 gene, and contain a single point mutation in the remaining allele at codons 273 and 194 respectively (Nigro *et al.*, 1989). However, the status of p53 in the biologically well-characterized cell line MCF 7 has not been determined. The p53 gene is expressed at elevated levels in these cells consistent with the presence of mutant p53 (Thompson *et al.*, 1990). However, immunohistochemical staining using monoclonal antibodies to the p53 protein show only reactivity against a minor population of MCF 7 cells (Bartek *et al.*, 1990a; 1990b).

To determine the status of the p53 gene in MCF 7 cells, we employed the methods of mRNA–cDNA synthesis and PCR amplification followed by asymmetric PCR and direct sequencing (Gyllensten & Erlich, 1988) (see Materials and methods for details). The vast majority of p53 gene mutations identified have clustered within a 'hot-spot' encompassing highly conserved regions of the gene (Nigro *et al.*, 1989). We therefore sequenced the region surrounding this 'hot-spot' starting with two independent mRNA isolations of MCF 7, using four overlapping sequence primers. We observed no mutations in the region encompassing exons 4–9 in either sample, and conclude that the cell line MCF 7 is wild-type for this region of the p53 gene.

The transfection of exogenous p53 genes into breast cancer cells

There were no differences in the total number of neomycin-resistant colonies arising from the transfection of either the mutant (pC53-SCX3) p53 gene or the vector pCMV-neo-Bam control in any of the three cell lines (data not shown). By comparison, there was a reduction of approximately 50% in the overall number of colonies arising following transfection of the wild-type (pC53-SN3) p53 gene into the MDA-MB 468 and T47 D cell lines (data not shown). In contrast, however, we did not observe any differences in the total number of MCF 7 colonies arising following transfection of the wild-type p53 gene. A second series of transfections confirmed these observations.

In the first experiment, neomycin-resistant colonies were clonally expanded and examined by Southern analysis for intact integration of the exogenous p53 gene. As shown in Table 1, a relatively small number of the MDA-MB 468 and T47 D clones contained intact exogenous wild-type p53 genes (0 of 9 and 1 of 8 clones analysed respectively). A second transfection experiment was undertaken to obtain a more significant assessment of the role of wild-type p53. To facilitate the analysis of a greater number of clones, transfectants were analysed at the colony stage by PCR analysis, using the P1 and P2 primers described previously (Nigro *et al.*, 1989). As described in the Materials and methods, colonies were transferred to two 48-well plates. DNA was extracted from a single well containing approximately 500–4000 cells and examined by PCR analysis for integration of the exogenous p53 gene. Clones which were positive in this assay were expanded for further analysis. As a control, a number of colonies which were negative by this assay were also expanded for Southern analysis. Repesentative Southern and PCR analyses are shown in Figure 1a and b respectively. The 1.8 kb exogenous p53 cDNA can be distinguished from the endogenous 7.8 kb p53 gene following digestion of genomic DNA with the restriction endonuclease BamHI. The p53 primers P1 and P2 specifically amplify a fragment of approximately 1.3 kb. In no case did we find non-concordance in our PCR and Southern hybridization data.

Table 1 Neomycin-resistant colonies containing intact exogenous p53 DNA*

Cell line	*Experiment no.*	*pC53-SN3 (wild-type)*	*pC53-SCX3 (mutant)*
MCF 7	1	5/32 (16%)	7/15 (47%)
	2	24/103 (23%)	18/40 (45%)
MDA-MB 468	1	0/9 (0%)	3/8 (38%)
	2	1/27 (4%)	8/41 (20%)
T47 D	1	1/8 (13%)	2/5 (40%)
	2	7/47 (15%)	4/11 (36%)

* Clones were regarded as positive if they contained exogenous p53 DNA of the correct size by Southern analysis (series 1) or by PCR analysis or both (series 2). PCR analyses were conducted on approximately 2×10^4 cells. Each transfection with wild-type p53 cDNA was performed in duplicate.

In most instances, between 36% and 47% of the neomycin-resistant clones which arose following transfection of the three cell lines with mutant p53 cDNA (pC53-SCX3) contained intact exogenous copies of this gene (Table 1). This contrasted greatly with the number of clones which contained intact exogenous wild-type p53 cDNA (pC53-SN3). In the case of the MDA-MB 468 and T47 D cell lines, the overall number of positive wild-type p53 clones was extremely small. Only 1 of 36 (3%) MDA-MB 468 clones, and 8 of 55 (15%) T47 D clones were positive. The number of positive MCF 7 wild-type p53 clones was higher, being 29 of 135 (22%). However, this does represent a reduction of nearly 50% compared with the number of positive mutant p53 clones (25 of 55, or 45%).

To determine the stability of transfectants, several MCF 7 mutant and wild-type p53 clones and MDA-MB 468 mutant p53 clones were maintained in continuous culture. Cell clones were grown for up to approximately 50 population doublings, and were analysed by PCR analysis. In no case did we find loss or rearrangement of the exogenous cDNA, confirming the stability of these clones (data not presented).

Expression studies

To determine whether or not the exogenous p53 genes were expressed, we examined all the MDA-MB 468 and T47 D wild-type p53 clones which contained an intact exogenous p53 gene. In addition, a number of mutant p53 transfectants and several MCF 7 wild-type and

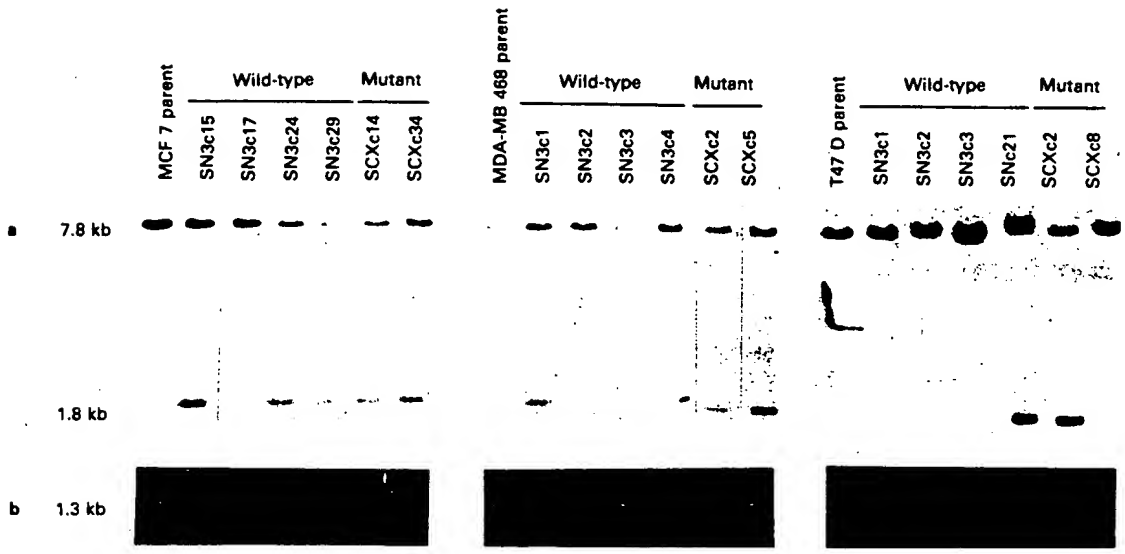

Figure 1 Southern and PCR analyses of p53 cDNA-transfected breast cancer cell lines. (a) Genomic DNA isolated from several p53 cDNA transfected clones was digested with the restriction endonuclease BamHI and hybridized with p53 cDNA. The endogenous p53 gene appears as a 7.8 kb fragment, and the exogenous cDNA as a 1.8 kb fragment. All lanes were loaded with 10 μg of DNA. (b) Analysis of the same clones by PCR amplification using the primers P1 and P2 (see Materials and methods), which amplify the entire coding region of the p53 gene. The 1.3 kb amplification product was electrophoresed on a 6% polyacrylamide gel and stained with ethidium bromide

mutant p53 transfectant clones were also examined. A representative Northern analysis is shown in Figure 2. The endogenous p53 gene appears as a 2.8 kb fragment, whereas the exogenous p53 gene appears as a 2.65 kb fragment. Data are summarized in Table 2. As can be seen, the majority of mutant p53 clones of all three cell lines expressed the exogenous gene at high levels. In contrast, the exogenous wild-type p53 gene was only expressed in MCF 7 transfectants and the single MDA-MB 468 wild-type p53 clone SN3c1 (see Figure 2). No T47 D transfectants expressed the exogenous wild-type p53 gene.

We examined two MCF 7 wild-type p53 clones (SN3c14 and SN3c29) to determine the effect of high expression of the exogenous wild-type gene upon cellular growth. Cells were plated at equal densities and compared with the parental MCF 7 cell line. There were no significant differences between the growth properties of the parental cell line compared with the two transfectants (see Figure 4a).

To examine the effect of a highly expressed wild-type p53 gene upon tumor growth, the MCF 7 clones SN3c24 and SN3c29 and MDA-MB 468 clone SN3c1 (see Figure 2) were injected into athymic nude mice and their growth properties compared directly with parental cells. Sites on five different animals were injected with 10^7 cells for each of the two MCF 7 clones, and sites on three mice were injected with 10^7 cells for the MDA-MB 468 clone. Parental cells were injected into the corresponding flank of the animals at the same cell inoculum. There was complete tumor take in all cases, and no difference in the growth rate of the developing tumors (data not shown).

These data suggest that wild-type p53 is not a suppressor of cellular growth in MCF 7 cells either *in vitro* or *in vivo*, as a signficant proportion of MCF 7 clones expressed elevated levels of the exogenous wild-type p53 gene. In contrast, this gene may function as a suppressor of cellular growth in breast cancer cells which contain only a single mutated copy of the p53 gene. This notion is supported by the observation that none of the T47 D clones which contained intact wild-type p53 genes was expressed. Indeed, the majority of wild-

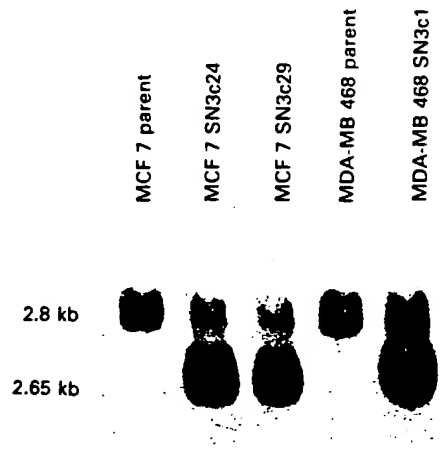

Figure 2 Northern analysis of total RNA from p53 transfectants hybridized with p53 cDNA. Endogenous p53 appears as a 2.8 kb transcript, whereas the exogenous gene appears below as a 2.65 kb transcript. Lanes 1 and 4 contain RNA from the parental cell lines MCF 7 and MDA-MB 468 respectively. Lanes 2 and 3 contain RNA from two representative wild-type p53 MCF 7 transfectants. Lane 5 contains RNA from the single wild-type p53 MDA-MB 468 transfectant SN3c1 which contained an intact exogenous p53 cDNA by Southern and PCR analyses

Table 2 Expression of p53 genes in breast cancer cells

	*Expressed clones/clones analysed**	
Cell line	*pC53-SN3*	*pC53-SCX3*
MCF 7	5/7	9/10
MDA-MB 468	1/1	5/6
T47 D	0/7	1/2

* Expression data of transfected clones previously shown to be positive by both PCR and Southern analyses

type p53 MDA-MB 468 transfectants also did not contain intact exogenous wild-type DNA sequences. However, a single clone (MDA-MB 468 SN3c1) did express wild-type p53 at high levels, and growth of this clone was unaffected both *in vitro* and *in vivo*. The role of the wild-type p53 gene as a suppressor of cellular growth would be substantially supported if we could show functional inactivation of this clone.

PCR amplification and sequencing

As a first step towards determining the status of the exogenously introduced gene in MDA-MB 468 SN3c1, we amplified genomic DNA by PCR using the primers P53CL1 and P53CL2. In this way we specifically amplified only the exogenous p53 cDNA, independent of the endogenous p53 gene sequences. The coding region encompassing exons 4–8 was sequenced using four internal primers which are described in Materials and methods. The exogenous p53 gene contained a C to A transition at codon 183, resulting in the conversion of a serine residue to a nonsense mutation (Figure 3). We confirmed this mutation following DNA sequencing in both orientations (data not shown). This mutation occurred near the mutation 'hot-spot' region of the p53 gene (Nigro *et al.*, 1989), and presumably must have arisen spontaneously either during DNA transfection or soon afterwards. This result confirms that no MDA-MB 468 transfectants expressed a wild-type p53 gene, thereby supporting the hypothesis that this gene is a suppressor of cellular growth in these cells.

Growth studies of p53 transfectants

There is compelling evidence supporting a dominant negative function for mutant p53 in murine cells (Michalovitz *et al.*, 1990). However, in the human system mutant p53 may not function in a similar way. Indeed Chen *et al.* (1990) found that the wild-type p53 gene was dominant when introduced along with a mutant p53 gene into the p53-null osteosarcoma cell line Saos-2 by retroviral transfer.

To examine the effect of elevated levels of mutant p53 upon cell growth, we compared the plating efficiencies and growth characteristics of several clones of MCF 7 and MDA-MB 468 which expressed exogenous mutant p53 at elevated levels. Figure 4 shows the plating efficiencies and growth of several clones compared with the parental cells. In no instance did we find any difference in the growth potential of either MCF 7 or MDA-MB 468 mutant p53 clones compared with the parental cells (Figure 4b and c respectively). In addition, we did not observe any differences in saturation density of these clones.

Figure 3 Position of p53 gene mutation in the transfectant MDA-MB 468 SN3c1. The C to A mutation at codon 183 is indicated, and results in the conversion of a serine residue to a stop codon

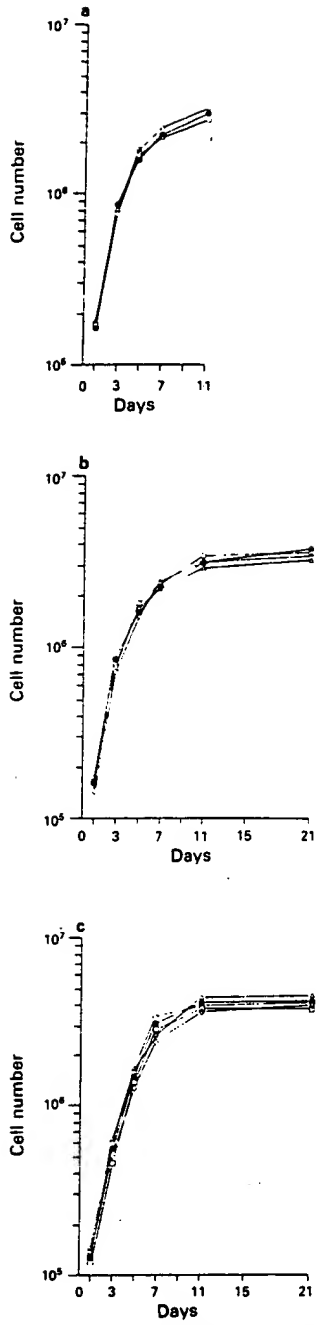

Figure 4 Growth rates and saturation densities of p53-transfected MCF 7 or MDA-MB 468 cells expressing wild-type or mutant exogenous p53 cDNA. (**a**) Growth rates of MCF 7 parental cells (●), transfectant clones MCF 7 SN3c14 (○) and MCF 7 SN3c29 (△), which express exogenous wild-type p53. (**b**) Growth rates of MCF 7 parental cells (●), transfectant clones MCF 7 SCXc30 (○), MCF 7 SCXc32 (△) and MCF 7 SCXc34 (◇), which express exogenous mutant p53. (**c**) Growth rates of MDA-MB 468 parental cells (●), transfectant clones MDA-MB 468 SCXc1 (○), MDA-MB 468 SCXc2 (△), MDA-MB 468 SCXc6 (◇) and MDA-MB 468 SCXc8 (□), which express exogenous mutant p53. All transfectant clones expressed exogenous p53 at high levels. Cells were fed every 2–3 days throughout the entire experiment

Discussion

An understanding of the genetic changes which occur in the transition of a normal cell to a tumor cell is crucial

if we are to develop new strategies towards both the prevention and treatment of cancer. Breast cancer is a particularly challenging disease. It is highly complex and ill-defined, and the natural history of the progression of primary breast cancer varies considerably from patient to patient. This heterogeneity makes patient management and therapeutic decisions difficult. Thus molecular approaches which will identify genetic subgroups would be invaluable in the overall management of this disease.

Mutations of the p53 gene have been identified in a significant proportion of breast tumors (Baker *et al.*, 1990; Bartek *et al.*, 1990b). We present evidence that the wild-type p53 gene functions as a suppressor of cellular growth in two breast cancer cell lines, MDA-MB 468 and T47 D, which harbor mutations in the p53 gene. Of 55 T47 D clones examined, none expressed exogenous wild-type p53, and only 1 of 36 MDA-MB 468 clones expressed this gene (compared with a significant proportion of which expressed the mutant p53 gene). This single MDA-MB 468 wild-type p53 cDNA transfectant was shown to contain a novel single nucleotide base mutation by DNA sequencing. We conclude that this clone was able to survive because of a spontaneous single nucleotide mutation which arose in the exogenous wild-type gene at the time of transfection or soon afterwards, resulting in the conversion of a serine residue to a stop codon. This is supported by the fact that MDA-MB 468 and T47 D cells were able to grow unaffected in the presence of elevated levels of an exogenous mutated p53 gene. Thus the only difference between the exogenous wild-type p53 gene (p53SCX3) and both the exogenous mutant gene used in these transfections and the spontaneous mutant which arose in MDA-MB 468SN3c1 is a change in a single nucleotide. This provides further evidence that only a subtle change is sufficient to alter the normal function of the wild-type p53 gene in a cell. The demonstration of suppression of cellular growth of breast cancer cells by wild-type p53 adds to a growing list of cancers, which includes colon cancer (Baker *et al.*, 1990), glioblastoma (Mercer *et al.*, 1990) and osteosarcoma (Chen *et al.*, 1990; Diller *et al.*, 1990), in which functional suppression has been demonstrated. These studies suggest that like the retinoblastoma gene, human p53 has a broad suppression activity in many different tumor types.

In contrast to MDA-MB 468 and T47 D, growth suppression by the wild-type p53 gene could not be demonstrated in the breast cancer cell line MCF 7. Although there was a reduction in the overall number of MCF 7 clones which expressed wild-type p53 compared with mutant p53, all the clones examined expressed the wild-type gene at elevated levels. That MCF 7 cells can grow unaffected in the presence of wild-type p53 means that it is highly unlikely that the growth suppression observed in the cell lines MDA-MB 468 and T47 D was simply due to a toxic effect. Further, it has been shown that the introduction by retroviral transfer of single copies of the wild-type p53 gene into osteosarcoma cells which do not express endogenous p53 is sufficient to suppress the neoplastic growth of these cells (Chen *et al.*, 1990).

Most analyses of mutation in the p53 gene have focused upon the highly conserved regions of the gene which are encompassed by the exons 4–9 (Iggo *et al.*, 1990; Malkin *et al.*, 1990; Rodrigues *et al.*, 1990), and we have shown by PCR amplification followed by direct DNA sequencing that MCF 7 cells are wild-type in this region. A small number of mutations have been described in other regions of the p53 gene (Vogelstein, 1990), and we cannot exclude the possibility that MCF 7 cells may contain a mutation in another region of the gene or promoter. If a mutation is present, however, this must presumably represent a functionally separate group from those found in the cell lines MDA-MB 468 and T47 D, as the introduction of the wild-type p53 gene into MCF 7 cells has no effect upon cellular growth. Functionally distinct mutations of the p53 gene have been described. For example, a clustering of p53 mutations has been observed in DNA from individuals with Li–Fraumeni syndrome (Malkin *et al.*, 1990; Srivastava *et al.*, 1990), and the mutant p53 proteins do not form complexes with the heat shock protein hsc 70 (Malkin *et al.*, 1990), as has been observed in other mutant p53 proteins (Finlay *et al.*, 1988). Further, a murine p53 variant has recently been described which contains a mutation adjacent to the cluster of p53 mutations seen in Li–Fraumeni individuals (Halevy *et al.*, 1990). This p53 mutant protein also does not bind hsc70 protein, and its transforming capabilities are severely reduced (Halevy *et al.*, 1990).

An alternative interpretation of these data is that MCF 7 cells reflect a group of tumors in which modifications in the p53 gene are not involved. Alterations in p53 have not been identified by either immunohistochemical means or by DNA sequencing in nearly 50% of breast tumors. In the progression of colon tumors, mutations rarely occur in the preneoplastic adenomatous stage (Baker *et al.*, 1989) and, interestingly, transfection of wild-type p53 has no effect upon the growth of these cells (Baker *et al.*, 1990). This result further supports the notion that the reintroduction of wild-type p53 into cells by DNA transfection is a valid assessment of the functional status of the p53 gene in these cells. Therefore the inability of wild-type p53 to suppress the growth of MCF 7 cells may be a true reflection of the lack of involvement of the p53 gene in the development of this disease. A molecular subclassification of breast tumors based upon the presence or absence of p53 mutations may prove to be invaluable. It remains to be determined, however, if this subclassification would have diagnostic or prognostic significance.

It has been proposed that mutant p53 may function in a dominant negative manner (Baker *et al.*, 1989; 1990). To assess a functional role for mutant p53 in breast cancer development, we examined several clones which expressed exogenous mutant p53 cDNA at high levels. No differences were observed in the plating efficiencies or growth rates of any clones compared with the parental cells. Interestingly, the introduction of mutant p53 by retroviral infection into the osteosarcoma cell line Saos-2 (which does not express endogenous p53) resulted in a greater saturation density (Chen *et al.*, 1990). In our experiments we did not observe an elevated saturation density in any clones examined. This discrepancy may be due to the fact that in the absence of wild-type p53 expression of mutant p53 *per se* has an effect upon saturation density, and that a further elevation of expression will have no further effect. Alternatively, either breast cancer cells may respond in different ways to mutant p53 gene

expression or different mutant p53 genes may have different effects upon saturation density. The last two suggestions could be addressed by introducing different mutant p53 genes into these cells.

Materials and methods

Cell culture

The breast cancer cell lines MCF 7, MDA-MB 468 and T47 D were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) with antibiotics, and maintained in a 5% CO_2 humidified atmosphere.

Transfection

Cells were plated at 70–80% confluency in 100 mm^2 dishes, and transfected with 15 $\mu g\,ml^{-1}$ plasmid DNA using the calcium phosphate procedure (Graham & van der Eb, 1973). Two separate plates were transfected with the wild-type p53 gene construct for each line, whereas only single plates were transfected with the mutated p53 gene and vector control. Following exposure to DNA in calcium phosphate for 4 h, cells were treated to a 15% glycerol shock for 1 min. After incubation overnight at 37°C each plate was trypsinized into five 100 mm^2 dishes and selection applied after 3 days of incubation. The MCF 7 and T47 D cell lines were exposed to 600 $\mu g\,ml^{-1}$ Geneticin (Gibco), whereas MDA-MB 468 cells were exposed to 300 $\mu g\,ml^{-1}$ Geneticin. Colonies arose after 14–21 days. The total number of colonies arising was determined after 4 weeks.

PCR analysis of clones

DNA was isolated either by conventional methods (Sambrook *et al.*, 1989), or by a rapid proteinase K/detergent method (Higuchi, 1989). The latter method enabled the analysis of neomycin-resistant clones containing approximately 500–4000 cells, which represents approximately 10–12 population doublings. Following selection in medium containing appropriate amounts of Geneticin, colonies were transferred to two 48-well plates. DNA was extracted by the proteinase K/detergent method and amplified using p53 primers P1 and P2 (described in Nigro *et al.*, 1989). Those clones which proved positive were expanded for further analysis.

Southern analysis

A 10 μg sample of DNA was digested with the restriction endonuclease BamHI following the manufacturer's instructions, and electrophoresed on 0.8% agarose gels. Southern transfer was performed using 0.4 M sodium hydroxide (Altherr *et al.*, 1989) and Genatran nylon membrane (Plasco). Hybridization probes were made from gel-purified (USBioclean) fragments using the random primer method of labeling (Feinberg & Vogelstein, 1983). The p53 probe used in these hybridization studies encompassed the entire cDNA.

Northern analysis

Total RNA was isolated by the acid–guanidinium thiocyanate method of Chomczynski & Sacchi (1987) with the modifications of Puissant & Houdebine (1990). Northern analyses were performed using 10–20 μg of total RNA. Following electrophoresis on 1.2% agarose/formaldehyde gels, RNA was transferred to GeneScreenPlus nylon membrane (Du Pont). Hybridization probes were made from gel-purified (USBioclean) fragments using the random primer method of labeling as described above.

PCR sequencing

Total RNA was isolated by the acid–guanidinium thiocyanate method described previously. Single-strand cDNA was generated using 1 μg of total RNA and 20 units of M-MLV reverse transcriptase (BRL) following the manufacturer's instructions. DNA was extracted from cell lines by conventional means (Sambrook *et al.*, 1989). Either 0.5 μg genomic DNA or one-fifth volume of the cDNA reaction was amplified using the following primers:

P53CL1 GCGGATCCACGACGGTGACACGCTTCCCTG
P53CL2 GCGGATCCGTCCTGGGTGCTTCTGACGCACAC

which contain additional BamHI sequences. Following amplification, samples were spun through Centricon 30 columns (Amicon) to remove excess nucleotides and primers, and a second, asymmetric IPCR amplification was performed (Gyllensten & Erlich, 1988; Allard *et al.*, 1991) using the following primers in a ratio of 50:1 (P53CL4:P53CL3):

P53CL3 CAGACTGCCTTCCGGGTCACCTG
P53CL4 GGAGGCTGTCAGTGGGGAACCC

Single-strand templates were purified using Centricon 30 columns, and sequenced by the dideoxy chain-termination method using a Sequenase 2.0 kit (United States Biochemicals) and the following primers:

P53SQ1 CCTGTCATCTTCTGTCCCTTCCCAG
P53SQ2 CTCCCCTGCCCTCAACAAG
P53SQ3 GGCCATCTACAAGCAGTC
P53SQ4 GTTGGCTCTGACTGTACCAC

The sequencing reaction mixes were separated by electrophoresis on gradient 6% polyacrylamide gels.

Plasmids

The plasmids used in these experiments have been described previously (Baker *et al.*, 1990). pCMV-neo-Bam is an expression vector containing the cytomegalovirus (CMV) LTR promoter, and the neomycin resistance gene under the control of the SV40 promoter–enhancer. Either a wild-type or a mutated p53 gene was introduced into this vector to create pC53-SN3 or pC53-SCX3 respectively. The only difference between the two constructs is a single nucleotide (T to C) change resulting in a substitution of alanine for valine at codon 143 in pC53-SCX3 (Baker *et al.*, 1990).

Cell growth studies

Cells were plated at a density of 4×10^5 cells/ml in 60 mm^2 dishes in duplicate. The data presented summarize three independent growth experiments. Cells were fed every 2 or 3 days.

Tumorigenicity assays

Cells were injected subcutaneously into 5- to 6-week-old athymic nude mice (Sprague Dawley) at inoculi of 10^7 cells per site. Mice were fed on sterile water containing 1 $\mu g\,ml^{-1}$ 17-alpha ethynylestradiol (Sigma), and monitored for tumor growth regularly. Tumors were allowed to develop for 28 weeks.

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References

Allard, M.W., Ellsworth, D.L. & Honeycutt, R.L. (1991). *BioTechniques*, **10**, 24–26.

Altherr, M.R., Smith, B., MacDonald, M.E., Hall, L. & Wasmuth, J.J. (1989). *Genomics*, **5**, 581–588.

Baker, S.J., Fearon, E.R., Nigro, J.M., Hamilton, S.R., Preisinger, A.C., Jessup, J.M., van Tuinen, P., Ledbetter, D.H., Barker, D.F., Nakamura, Y., White, R. & Vogelstein, B. (1989). *Science*, **244**, 217–221.

Baker, S.J., Markowitz, S., Fearon, E.R., Willson, J.K.V. & Vogelstein, B. (1990). *Science*, **249**, 217–221.

Bartek, J., Bartkova, J., Vojtesek, B., Staskova, Z., Rejthar, A., Kovarik, J. & Lane, D.P. (1990a). *Int. J. Cancer*, **46**, 839–844.

Bartek, J., Iggo, R., Gannon, J. & Lane, D.P. (1990b). *Oncogene*, **5**, 893–899.

Cattoretti, G., Rilke, F., Andreola, S., D'Amato, L. & Delia, D. (1988). *Int. J. Cancer*, **41**, 178–183.

Chen, P.-L., Chen, Y., Brookstein, R. & Lee, W.-H. (1990). *Science*, **250**, 1576–1580.

Chomczynski, P. & Sacchi, N. (1987). *Anal. Biochem.*, **162**, 156–159.

Coles, C., Thompson, A.M., Elder, P.A., Cohen, B.B., Mackenzie, I.M., Cranston, G., Chetty, U., Mackay, J., Macdonald, M., Nakamura, Y., Hoyheim, B. & Steel, C.M. (1990). *Lancet*, **336**, 761–763.

Crawford, L.V., Pim, D. & Lamb, P. (1984). *Mol. Biol. Med.*, **2**, 261–272.

Devilee, P., van den Broek, M., Kuipers-Dijkshoorn, N., Kolluri, R., Meera Khan, P., Pearson, P.L. & Cornelisse, C.J. (1989). *Genomics*, **5**, 554–560.

Devilee, P., Cornelisse, C.J., Kuipers-Dijkshoorn, N., Jonker, C. & Pearson, P.L. (1990). *Cytogenet. Cell Genet.*, **53**, 52–54.

Diller, L., Kassel, J., Nelson, C.E., Gryka, M.A., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S.J., Vogelstein, B. & Friend, S.H. (1990). *Mol. Cell Biol.*, **10**, 5772–5781.

Eliyahu, D., Raz, A., Gruss, P., Givol, D. & Oren, M. (1984). *Nature*, **312**, 646–649.

Eliyahu, D., Goldfinger, N., Pinhasi-Kimhi, O., Shaulsky, G., Skurnik, Y., Arai, N., Rotter, V. & Oren, M. (1988). *Oncogene*, **3**, 313–321.

Feinberg, A.P. & Vogelstein, B. (1983). *Anal. Biochem.*, **132**, 6–13.

Finlay, C.A., Hinds, P.W., Tan, T.-H., Eliyahu, D. & Oren, M. (1988). *Mol. Cell. Biol.*, **8**, 531–539.

Finlay, C.A., Hinds, P.W. & Levine, A. (1989). *Cell*, **57**, 1083–1093.

Graham, F.L. & van der Eb, A.J. (1973). *Virology*, **52**, 456.

Gyllensten, U.B. & Erlich, H.A. (1988). *Proc. Natl. Acad. Sci. USA*, **85**, 7652–7656.

Halevy, O., Michalovitz, D. & Oren, M. (1990). *Science*, **250**, 113–116.

Higuchi, R. (1989). *Amplifications*, **2**, 1–3.

Hinds, P., Finlay, C. & Levine, A.J. (1989). *J. Virol.*, **63**, 739–746.

Iggo, R., Gatter, K., Bartek, J., Lane, D. & Harris, A.L. (1990). *Lancet*, **335**, 675–679.

Isobe, M., Emanuel, B.S., Givol, D., Oren, M. & Croce, C.M. (1986). *Nature*, **320**, 84–85.

Jenkins, J.R., Rudge, K. & Currie, G.A. (1984). *Nature*, **312**, 651–654.

Lane, D.P. & Benchimol, A. (1990). *Genes Dev.*, **4**, 1–8.

Lane, D.P. & Crawford, L.V. (1979). *Nature*, **278**, 261–263.

Li, F.P. & Fraumeni, J.F. (1969). *Ann. Intern. Med.*, **71**, 747–751.

McBride, O.W., Merry, D. & Givol, D. (1986). *Proc. Natl. Acad. Sci. USA*, **83**, 130–134.

Mackay, J., Steel, C.M., Elder, P.A., Forrest, A.P.M. & Evans, H.J. (1988). *Lancet*, **ii**, 1384–1385.

Malkin, D., Li, F.P., Strong, L.C., Fraumeni, J.F., Nelson, C.E., Kim, D.H., Kassel, J., Gryka, M., Bischoff, F.Z., Tainsky, M.A. & Friend, S.H. (1990). *Science*, **250**, 1233–1238.

Mercer, W.E., Shields, M.T., Amin, M., Sauve, G.J., Appella, E., Romano, J.W. & Ullrich, S.J. (1990). *Proc. Natl. Acad. Sci. USA*, **87**, 6166–6170.

Michalovitz, D., Halevy, O. & Oren, M. (1990). *Cell*, **62**, 671–680.

Mulligan, L.M., Matlashewski, G.J., Scrable, H.J. & Cavenee, W.K. (1990). *Proc. Natl. Acad. Sci. USA*, **87**, 5863–5867.

Nigro, J.M., Baker, S.J., Preisinger, A.C., Jessup, J.M., Hostetter, R., Cleary, K., Bigner, S.H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F.S., Weston, A., Modali, R., Harris, C.C. & Vogelstein, B. (1989). *Nature*, **342**, 705–708.

Parada, L.F., Land, H., Weinberg, R.A., Wolf, D. & Rotter, V. (1984). *Nature*, **312**, 649–651.

Prosser, J., Thompson, A.M., Cranston, G. & Evans, H.J. (1990). *Oncogene*, **5**, 1573–1579.

Puissant, C. & Houdebine, L.-M. (1990). *BioTechniques*, **8**, 148–149.

Rodrigues, N.R., Rowan, A., Smith, M.E.F., Kerr, I.B., Bodmer, W.F., Gannon, J.V. & Lane, D.P. (1990). *Proc. Natl. Acad. Sci, USA*, **87**, 7555–7559.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York.

Srivastava, S., Zou, Z., Pirollo, K., Blattner, W. & Chang, E.H. (1990). *Nature*, **348**, 747–749.

Takahashi, T., Nau, M.M., Chiba, I., Birrer, M.J., Rosenberg, R.K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A.F. & Minna, J.D. (1989). *Science*, **246**, 491–494.

Thompson, A.M., Steel, C.M., Chetty, U., Hawkins, R.A., Miller, W.R., Carter, D.C., Forest, A.P.M. & Evans, H.J. (1990). *Br. J. Cancer*, **61**, 74–78.

Varley, J.M., Brammar, W.J., Lane, D.P., Swallow, J.E., Dolan, C. & Walker, R.A. (1991). *Oncogene*, **5**, 413–421.

Vogelstein, B. (1990). *Nature*, **348**, 681–682.

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Inhibition of Viral and Cellular Promoters by Human Wild-Type p53

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Mutation of the p53 tumor suppressor gene is a recurring event in a variety of human cancers. Wild-type p53 may regulate cell proliferation and has recently been shown to repress transcription from several cellular promoters. We studied the effects of wild-type and mutant human p53 on the human proliferating-cell nuclear antigen promoter and on several viral promoters including the simian virus 40 early promoter-enhancer, the herpes simplex virus type 1 thymidine kinase and UL9 promoters, the human cytomegalovirus major immediate-early promoter-enhancer, and the long terminal repeat promoters of Rous sarcoma virus, human immunodeficiency virus type 1, and human T-cell lymphotropic virus type I. HeLa cells were cotransfected with a wild-type or mutant p53 expression vector and plasmids containing a chloramphenicol acetyltransferase reporter gene under viral (or cellular) promoter control. Expression of wild-type p53 correlated with a consistent and significant (6- to 76-fold) reduction of reporter enzyme activity. A mutation at amino acid 143 of p53 releases this inhibition significantly with all the promoters studied. Expression of a p53 mutated at any one of the five amino acid positions 143, 175, 248, 273, and 281 also correlated with a much smaller (one- to sixfold) reduction of reporter enzyme activity from the herpes simplex virus type 1 thymidine kinase promoter. These mutant forms of p53 are found in various cancer cells. Thus, failure of tumor suppression correlates with loss of the promoter inhibitory effect of p53.

p53 is a nuclear phosphoprotein that was initially detected in association with simian virus 40 (SV40) large T antigen in virus-transformed rodent cells (31, 33). Elevated levels of p53 were subsequently observed in cell lines transformed by a variety of agents, including DNA and RNA tumor viruses, irradiation, and chemical carcinogens (13, 16, 24, 29, 35, 48). When genomic and cDNA clones of p53 were found to immortalize primary cells and to cooperate with the *ras* oncogene in transformation of primary cells, p53 was consigned to the nuclear oncogene family of *myc* and *myb* (16, 29, 45); only recently has it been learned that the original clones contained activating mutations (24). Expression of wild-type p53 has now been shown to inhibit proliferation of transformed cells, suppress oncogene-mediated cell transformation, and eliminate the tumorigenic potential of tumor-derived cell lines (2, 3, 8, 9, 14–16, 18, 36, 38, 40). Like the retinoblastoma susceptibility (RB) gene, p53 is now considered to be an antioncogene or tumor suppressor gene (see reference 32 for a review). Somatic and germ line (in Li-Fraumeni syndrome) mutation of the p53 gene has been detected in a variety of human tumors, with mutations concentrated in phylogenetically conserved sequence domains (26, 32, 34, 54). At present, p53 mutations are the most frequently reported genetic defects in human cancer (3, 26, 27, 44, 56, 58).

Several biochemical functions are attributed to p53. p53-GAL4 fusion proteins can activate transcription from promoters containing GAL4-binding sites, suggesting that p53 is a transactivator (17, 47). Moreover, sequence-specific DNA binding by p53 has been reported (4, 30). Wild-type (but not mutant) p53 binds to the 21-bp repeats of the SV40 early and late promoters (4) and to TGCCT repeats present in the human ribosomal gene cluster (30). p53 inhibits SV40 DNA replication in vivo and in vitro by complexing with T antigen and inhibiting the unwinding capability of T antigen (5, 19, 20, 59). Wild-type p53 has recently been shown to inhibit c-*fos* transcription (21) and to repress transcription from several cellular promoters (10, 21, 50).

The human proliferating-cell nuclear antigen (PCNA) gene is growth regulated (1, 28) and encodes a protein that is a component of the DNA replication machinery of the cell. PCNA has been identified as a cofactor of DNA polymerase δ (6, 46, 55). Mercer et al. (39) demonstrated a down-regulation of PCNA mRNA and protein by wild-type p53. However, the mechanism of this regulation was not known since the possibility that p53 might affect the PCNA promoter activity was not examined.

Several viruses have mechanisms to target (and presumably inactivate) wild-type p53 by their transforming proteins. SV40 large T antigen, adenovirus 5 E1B, and E6 of human papillomavirus (HPV) types 16 and 18 bind specifically to p53 and either sequester it (large T, E1B) or promote its degradation (E6) (31, 33, 51, 52, 60). The effect of p53 on promoter activity of viruses has not been investigated in detail.

We studied the effect of wild-type and mutant human p53 expression on the activity of PCNA and several viral promoters fused to a chloramphenicol acetyltransferase (CAT) reporter gene. Expression of wild-type p53 correlated with a consistent and significant (6- to 76-fold) inhibition of reporter enzyme activity in HeLa cells. Significantly, mutants of p53 found in cancer cell lines exert this inhibitory effect on the promoter function in this assay. This suggests that the promoter inhibitory activity of p53 is crucial for its tumor suppressor activity.

MATERIALS AND METHODS

DNA plasmids. Wild-type and mutant human p53 expression plasmids (generously provided by Arnold J. Levine) utilize the human cytomegalovirus (HCMV) major immedi-

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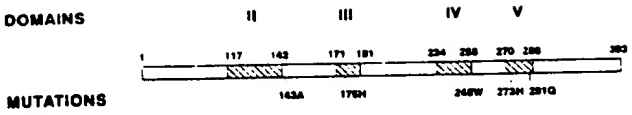

FIG. 1. Schematic representation of the p53 gene product. Conserved domains II to V are indicated by hatched areas. Positions of amino acid substitutions in the mutants that are used in this study are indicated below.

ate-early promoter-enhancer (−671 to +73) in the vector pHCMV-Neo-Bam (25). p53-cWT contains a wild-type p53 cDNA, while p53-c143A (Val to Ala at amino acid 143) and p53-c248W (Arg to Trp at amino acid 248) contain mutant p53 cDNAs (25). p53-175H (Arg to His at amino acid 175), p53-273H (Arg to His at amino acid 273), and p53-281G (Asp to Gly at amino acid 281) are mutant p53 cDNA-genomic chimeras, all containing introns 2 through 4 (25). The neomycin resistance gene was removed from all plasmids by treatment with *Hin*dIII and *Xba*I.

The CAT plasmids described here all contain the *Escherichia coli* CAT gene under the transcriptional control of the following promoters: PCNA (human PCNA promoter) (41); pSV2 (SV40 early promoter-enhancer) (22); CMV (HCMV major immediate-early promoter-enhancer) (11); HSV-1.TK (herpes simplex virus type 1 thymidine kinase promoter) (37); UL9 (HSV-1 UL9 gene promoter [12a]); RSV (Rous sarcoma virus 3′ long terminal repeat [LTR]) (12); HIV-1 (human immunodeficiency virus type 1 LTR) (43); and HTLV-I (human T-cell lymphotropic virus type I LTR) (53). The plasmids are designated promoter name.CAT. PCNA.CAT was generously provided by Gilbert Morris.

Cell culture and transfection. Human cervical carcinoma (HeLa) and monkey kidney (Vero) cells were obtained from the American Type Culture Collection and propagated in minimum essential medium containing 10% fetal calf serum and Dulbecco's minimum essential medium, respectively. Subconfluent cells were transfected by the calcium phosphate-DNA coprecipitation method with a dimethyl sulfoxide shock 4 h posttransfection (8, 23). In a typical experiment, 5×10^6 cells were cotransfected with 2.5 μg of a reporter gene construct and 5 μg of a p53 expression plasmid (or 5 μg of the expression vector without p53 sequences as a control). All transfection experiments were repeated several times.

CAT assay. Cells were harvested 48 h posttransfection and lysed by three successive cycles of freezing and thawing. Extracts were normalized for protein concentration and assayed for CAT enzyme activity (22). CAT activity was detected by thin-layer chromatographic separation of [^{14}C]chloramphenicol from its acetylated derivatives and quantitated by cutting out radioactive spots from the thin-layer chromatograph plate after autoradiography.

Metabolic labeling and immunoprecipitation. At 48 h after transfection with 20 μg of wild-type or mutant human p53 expression plasmids (or expression vector pHCMV-Bam), HeLa cells were incubated in methionine-free minimal essential medium for 20 min and subsequently metabolically labeled for 4 h with [^{35}S]methionine (ICN Tran[^{35}S]-label) at 70 μCi/ml in methionine-free minimal essential medium (49). Cells were lysed, and extract aliquots were immunoprecipitated with PAb421, a cross-species, carboxy-terminal-specific, anti-p53 monoclonal antibody (p53 Ab-1; Oncogene Science) (57), and protein A-agarose (Calbiochem). Immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography.

RESULTS

Expression of wild-type and mutant human p53 proteins in transfected HeLa cells. We used wild-type and mutant human p53-expressing clones for our analysis of the effect(s) of p53 on the function of various promoters. The mutants were c143A, 175H, c248W, 273H, and 281G, where capital letters indicate mutant amino acids and small c indicates cDNA clones. These mutants were chosen because they contain the frequently mutated amino acid residues found in tumors (26) (Fig. 1). These residues fall in or near four domains (II to V) which are highly conserved in vertebrate species (54).

To determine whether the mutants and wild-type proteins were expressed, we transfected HeLa cells with wild-type or mutant p53 expression plasmids or with expression vector pHCMV.Bam (vector alone) and metabolically labeled them with [^{35}S]methionine. Cell lysates were immunoprecipitated with PAb421, a cross-species, carboxy-terminal-specific, anti-p53 monoclonal antibody, and protein A-agarose. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by autoradiography (Fig. 2). Transfection of HeLa cells with either wild-type or mutant p53 expression plasmids led to the specific immunoprecipitation of proteins migrating at an approximate molecular weight of 53,000 (indicated by an arrowhead), while transfection with the expression vector did not. Mutant p53 proteins appear to be expressed at higher levels than wild-type in transfected HeLa cells. This is consistent with the extended half-life of mutant p53 proteins (32). This effect would also be due to inhibitory effects exerted by wild-type p53 on the CMV promoter. Mutant proteins would not be as inhibitory, resulting in a higher level of expression. In the lanes containing mutant p53s (c143A, 175H, and 281G), a band at about 70 kDa is visible. This band may indicate complex formation between mutant p53s and the cellular heat shock protein 70 (25, 32). We do not know the identity of the 18-, 43-, and 200-kDa bands seen in all lanes, including vector alone. The immunoprecipitation results shown in Fig. 2 clearly indicate successful expression of wild-type and mutant human p53s in HeLa cells after transfection with the corresponding expression plasmids.

Modulation of PCNA promoter activity by wild-type and

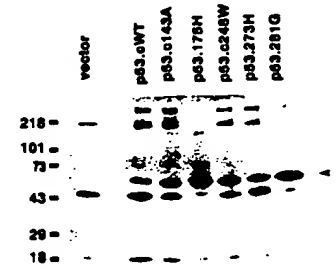

FIG. 2. Expression of wild-type and mutants of human p53 by transfection of expression plasmids into HeLa cells. HeLa cells were transfected with pHCMV.Bam expression vector alone and wild-type or mutant human p53 expression plasmid DNA; the proteins were then metabolically labeled with [^{35}S]methionine and immunoprecipitated with p53-specific monoclonal antibody as described in Materials and Methods. Immunoprecipitates were analyzed on an SDS-polyacrylamide gel. The arrowhead shows bands corresponding to p53. Numbers on left show sizes in kilodaltons.

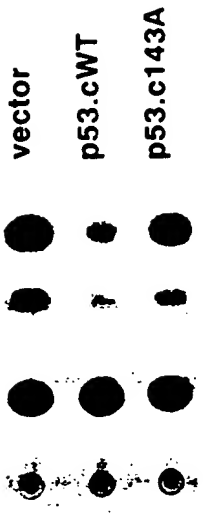

FIG. 3. Effect of wild-type and a mutant human p53 on the expression of PCNA.CAT in HeLa cells. Subconfluent HeLa cells were cotransfected with PCNA.CAT (2.5 μg) and pHCMV.Bam (vector alone) or pHCMV.Bam expressing either wild-type or a mutant p53 (143 V→A), using the calcium phosphate precipitation technique as described in Materials and Methods. At 48 h posttransfection, cells were harvested and a CAT assay was done as described in the text. Experiments were repeated several times with similar qualitative results; one representative example is shown.

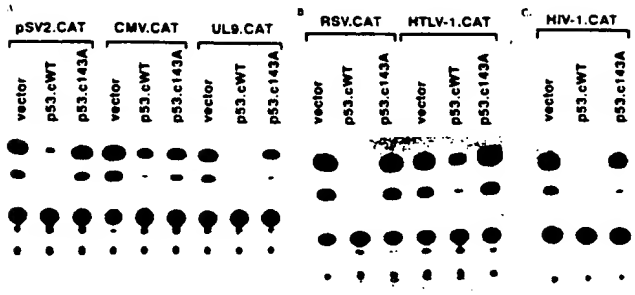

FIG. 4. Effect of wild-type and a mutant human p53 on the expression of viral promoter-CAT constructs in HeLa cells. The promoter-CAT constructs indicated (see text) were transfected separately into HeLa cells along with pHCMV.Bam (vector alone) or pHCMV.Bam expressing either wild-type or mutant p53 (143 V→A) as described in the legend to Fig. 3. For pSV2.CAT, 0.5 μg of the CAT plasmid was used with 5 μg of vector or p53 expression plasmid. All others were used as described in Materials and Methods.

mutant human p53. The PCNA gene encodes a nuclear protein that acts as an auxiliary factor of DNA polymerase δ and is presumably a part of the cellular replication machinery (55). It has been shown previously (39) that growth suppression induced by wild-type p53 protein is accompanied by a down-regulation of PCNA expression. Therefore, we were interested in determining whether wild-type p53 can inhibit the function of the PCNA promoter and, if so, whether a mutant p53 can exert the same effect. PCNA.CAT (41) was cotransfected into HeLa cells by the calcium phosphate precipitation technique as described in Materials and Methods with the pHCMV.Bam expression vector alone or with the plasmid expressing either the wild-type or the mutant (c143A) form of p53. After 48 h, CAT activity (PCNA promoter activity) was assayed in these cells. Wild-type p53 inhibited PCNA.CAT activity in transient assays by more than sixfold, while the mutant inhibited activity by one- to twofold (Fig. 3). Thus, the promoter inhibition is due to wild-type p53, and a mutation in the p53 gene destroys the inhibitory effect.

Effect of expression of wild-type and mutant p53 on various viral promoters. To analyze the effect of expression of wild-type and mutant p53 on various viral promoters, we used the following promoter-CAT constructs: SV40 early promoter (pSV2.CAT) (22), CMV early promoter-enhancer (CMV.CAT) (11), HSV-1 UL9 promoter (UL9.CAT) (13a), HIV-1 LTR (HIV.CAT) (43), RSV LTR (RSV.CAT) (12), and HTLV-I LTR (HTLV.CAT) (53). The promoter activities were determined by CAT assay after cotransfecting the respective promoter constructs with the pHCMV.Bam expression vector alone or with the plasmid expressing either the wild-type or a mutant (c143A) form of p53 into HeLa cells (Fig. 4; Table 1). The experiments were repeated several times with qualitatively similar results. Representative examples are shown in Fig. 4. All the promoters were inhibited significantly by the expression of wild-type p53. On the other hand, the mutant p53 had a relatively minor, if any, effect on expression of the various promoter-CAT constructs. In most of the cases, although the inhibition persisted with the mutant p53 (c143A), its extent was greatly reduced. In at least one case (HTLV-.CAT), the mutant actually stimulated the activity about 50%. This is not entirely surprising since recently Chin et al. (10) reported that the human multi-drug-resistant (*MDR*1) gene promoter is activated by another mutant p53 (175H).

All the promoters examined were inhibited by wild-type human p53, albeit to different extents. SV40 early promoter seems to be least affected under our assay conditions. To observe a significant extent of inhibition, we had to lower the pSV2.CAT concentration to 0.5 μg per transfection. The difference in the extent of inhibition by the same amount of wild-type p53 expression construct indicates that the observed promoter inhibition is possibly not an effect of general lethality caused by p53.

Effect of expression of wild-type and various mutants of human p53 on HSV-1 TK gene promoter. To determine the effect of other mutant p53 proteins on promoter activity, we tested wild-type and various mutants of human p53 with the HSV-1 TK gene promoter-CAT construct TK.CAT. The mutants of human p53 chosen for this study were described above and are as follows: p53-c143A, p53-175H, p53-c248W, p53-273H, and p53-281G. As shown in Fig. 5, TK promoter activity was inhibited most dramatically by wild-type p53, while the mutants inhibited to different extents. It is clear

TABLE 1. Inhibition of activity of different promoters by human wild-type (WT) p53 and mutant c143A relative to vector alone in HeLa cells

Promoter	Activity relative to vector alone (%)	
	WT p53	c143A p53
PCNA	15.9	58.8
RSV LTR	1.3	76.9
HTLV-I LTR	14.3	156.3
HIV LTR	2.3	47.6
UL9 (HSV)	2.8	31.2
SV40 early promoter	6.1	90.9
CMV early promoter	7.2	16.1

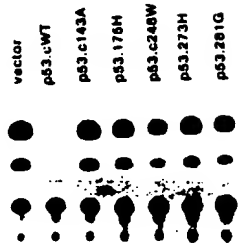

FIG. 5. Effect of expression of different mutant human p53s on the expression of HSV-1 TK promoter activity. HeLa cells were cotransfected with TK.CAT and pHCMV.Bam (vector alone) or pHCMV.Bam expressing either wild-type (cWT) or one of the mutant p53s: c143A (V to A at amino acid 143), 175H (R to H at amino acid 175), c248W (R to W at amino acid 248), 273H (R to H at amino acid 273), and 281G (D to G at amino acid 281) as described in the text. CAT assays were done as described in the text.

that all the mutants tested show a dramatic loss in mediating inhibition of the promoter. The failure of tumor suppression by these mutant p53 proteins correlates with the loss of the promoter inhibitory effect.

Inhibition of activity of various promoters by wild-type human p53 in Vero cells. To determine whether the p53-mediated promoter inhibition is cell type specific or is influenced by the expression of E6 of HPV 18 in the HeLa cell line, we chose also to use a monkey kidney cell line (Vero). Table 2 shows the percentage of acetylation of [^{14}C]chloramphenicol with various promoters in the presence and absence of wild-type human p53. The results indicate that in the Vero cell line (a nontransformed cell line) also, wild-type human p53 significantly inhibits various promoter activities (6- to 28-fold).

DISCUSSION

The results described above show that overexpression of wild-type human p53 can exert an inhibitory effect on a variety of viral promoters as well as on the cellular PCNA promoter (6- to 76-fold, Table 1). Several other groups recently reported an inhibitory activity of p53 on different cellular promoters. Santhanam et al. (50) found that wild-type p53 inhibited the promoters for interleukin 6, c-*fos*, beta-actin, and the porcine major histocompatibility complex class I gene. Ginsberg et al. (21) described the inhibition of c-*fos*, beta-actin, p53, hsc70, and c-*jun* promoters, while Chin et al. (10) showed that the *MDR*1 gene promoter was inhibited by p53. Combining our results with those reported previously, it becomes clear that a wide variety of cellular and viral promoters are inhibited by wild-type human p53. In all the cases, mutant p53 proteins found in tumors were either less inhibitory or in some cases stimulatory (10) (Fig. 4) for the promoter function. The extent of inhibition by wild-type p53 appears to depend on the promoter tested (Table 1); for example, the CMV promoter (CMV.CAT) was not inhibited to the same extent as the HIV LTR promoter (HIV.CAT). The SV40 early promoter seems to be least affected under our assay conditions. To observe a significant extent of inhibition, we had to lower the pSV2.CAT concentration to 0.5 μg per transfection. The difference in the extent of inhibition by the same amount of wild-type p53-expressing construct indicates that the observed promoter inhibition is possibly not an effect of general lethality caused by p53. Also, different mutants of p53 have different quantitative effects on promoter inhibition (Fig. 5; Table 3). Despite these differences, it is both interesting and significant that such a wide variety of viral promoters are inhibited by p53. What effect endogenous p53 has on these promoters in the course of viral infection is not known. It remains to be seen whether all these viruses may have molecular mechanisms to circumvent p53 inhibition. This may represent a unique strategy to allow viral replication not previously defined for nononcogenic viruses, whereas alteration of p53 by tumor viruses such as SV40, adenovirus, and HPV 16 and 18 has been established (31, 33, 51, 52, 60).

TABLE 2. Inhibition of different promoters by human wild-type p53 relative to vector alone in Vero cells

Promoter	Activity relative to vector alone (%)	Fold inhibition
PCNA	15.4	6.5
RSV LTR	3.5	28.6
HIV LTR	10.3	9.7
UL9 (HSV)	11.1	9.0
CMV	12.5	8.0

TABLE 3. Inhibition of HSV-1 TK promoter by human wild-type p53 and different mutants

p53	Activity relative to vector alone (%)
cWT	1.1
c143A	66.7
175H	37.0
c248W	14.5
273H	37.0
281G	21.2

Most of our experiments were performed in HeLa cells, which are known to have the E6 protein of HPV 18. Since HPV 18 E6 protein is known to interact with p53, a possibility remains that the data observed were influenced by this interaction. However, we also observed significant promoter inhibition in Vero cells (a nontransformed cell line) (Table 2). This suggests that the promoter inhibition is probably because of p53 alone.

Because p53 possesses the promoter inhibitory activity, it is possible that at least one of the mechanisms by which wild-type p53 inhibits cellular proliferation is by inhibiting cellular promoters. This is based on the assumption that p53 directly inhibits transcriptional activity. This remains to be determined by using in vitro transcription systems. However, the fact that the inhibitory effect of p53 is exerted on a wide variety of promoters, both cellular and viral, suggests that p53 probably affects one or more of the common generalized transcription factors or that it binds to promoter sequences nonspecifically and inhibits transcription. At this stage, we should also be aware of the possibility that the observed promoter inhibition is an effect of wild-type p53 when it is overexpressed. Under normal conditions, such a high concentration of p53 is not expected. However, it is not difficult to imagine that at a certain point in the cell cycle, local effective concentration of p53 may rise high enough to modulate cellular promoter activities.

p53 remains an extremely important and intriguing molecule. It has been demonstrated that purified wild-type p53 can bind to cellular DNA (30) as well as the SV40 early promoter region (4). It has also been shown that it can

function as a transcriptional activator when expressed as a chimera with the GAL4 DNA-binding domain on promoters with GAL4 DNA-binding sites (17, 47). The same molecule may function as an activator as well as an inhibitor of transcription. It is tempting to speculate that while p53 acts as a generalized inhibitor of transcription, it could activate certain promoters where it can bind effectively. We have observed that it requires a relatively higher concentration of p53 plasmid to inhibit the SV40 early promoter, which has p53-binding sites (4). One can speculate that p53 may exert its tumor suppressor function in several ways. First, under certain conditions, p53 may inhibit genes required for progression through the cell cycle. The inhibition of the PCNA promoter demonstrated in this study supports this mechanism. In addition, p53 may also activate expression of genes involved in the regulation of normal cell cycle progression. This regulation of expression may require the presence of p53-binding sites as *cis*-acting factors at the target gene. A possibility also exists that p53 activates the production of a factor that interacts with the transcription machinery and inhibits gene expression. Thus, p53 may act as a central factor in controlling the dynamic pattern of gene expression required for maintenance of a normal cell cycle. Both the tumor suppressor gene products RB and p53 have cellular antiproliferative activity. In one way, at least, they have a similar biochemical function—inhibition of transcription. It has been suggested that at least one mechanism by which RB may inhibit specific transcriptional activity is by complexing with the transcription factor E2F (7, 42). While it is not yet clear how p53 exerts its effect, similarity in biochemical function is an intriguing common theme.

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REFERENCES

1. **Almendral, J. M., D. Huebsch, A. P. Blundell, H. MacDonald-Bravo, and R. Bravo.** 1987. Cloning and sequence of the human nuclear protein cyclin: homology with DNA-binding proteins. Proc. Natl. Acad. Sci. USA **84**:1575–1579.
2. **Baker, S. J., E. R. Fearon, J. M. Nigro, S. R. Hamilton, A. C. Preisinger, J. M. Jessup, P. Van Tuizen, D. H. Ledbetter, D. F. Barker, Y. Nakamura, R. White, and B. Vogelstein.** 1989. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science **244**:217–221.
3. **Baker, S. J., K. Markowitz, E. R. Fearon, J. K. V. Willson, and B. Vogelstein.** 1990. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science **249**:912–915.
4. **Bargonetti, J., P. N. Friedman, S. E. Kern, B. Vogelstein, and C. Prives.** 1991. Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication. Cell **65**:1083–1091.
5. **Braithwaite, A. W., H. W. Sturzbecher, C. Addison, C. Palmer, K. Rudge, and J. R. Jenkins.** 1987. Mouse p53 inhibits SV40 origin-dependent DNA replication. Nature (London) **329**:458–460.
6. **Bravo, R., R. Frank, A. P. Blundell, and H. MacDonald-Bravo.** 1987. Cyclin/PCNA is the auxiliary protein of DNA polymerase δ. Nature (London) **326**:515–517.
7. **Cao, L., B. Faha, M. Dembski, L.-H. Tsai, E. Harlow, and N. Dyson.** 1992. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. Nature (London) **355**:176–179.
8. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7**:2745–2752.
9. **Chen, P.-L., Y. Chen, R. Bookstein, and W.-H. Lee.** 1990. Genetic mechanisms of tumor suppression by the human p53 gene. Science **250**:1576–1580.
10. **Chin, K.-V., K. Ueda, I. Pastan, and M. M. Gottesman.** 1992. Modulation of activity of the promoter of the human *MDR*1 gene by *ras* and p53. Science **255**:459–462.
11. **Cullen, B. R.** 1986. *Trans*-activation of human immunodeficiency virus occurs via a bimodal mechanism. Cell **46**:973–982.
12. **Cullen, B. R., P. T. Lomedico, and G. Ju.** 1984. Transcriptional interference in avian retroviruses—implications for the promoter insertion model of leukaemogenesis. Nature (London) **307**:241–245.

12a. **Deb, S. P.** Unpublished data.

13. **DeLeo, A. B., G. Jay, E. Appella, G. C. Dubois, L. W. Law, and L. J. Old.** 1979. Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. Proc. Natl. Acad. Sci. USA **76**:2420–2424.
14. **Diller, L., J. Kassel, C. E. Nelson, M. A. Gryka, G. Litwak, M. Gebhardt, B. Bressac, M. Ozturk, S. J. Baker, B. Vogelstein, and S. H. Friend.** 1990. p53 functions as a cell cycle control protein in osteosarcomas. Mol. Cell. Biol. **10**:5772–5781.
15. **Eliyahu, D., D. Michalovitz, S. Eliyahu, O. Pinhasi-Kimhi, and M. Oren.** 1989. Wild-type p53 can inhibit oncogene-mediated focus formation. Proc. Natl. Acad. Sci. USA **86**:8763–8767.
16. **Eliyahu, D., A. Raz, P. Gruss, D. Givol, and M. Oren.** 1984. Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. Nature (London) **312**:646–649.
17. **Fields, S., and S. K. Jang.** 1990. Presence of a potent transcription activating sequence in the p53 protein. Science **249**:1046–1049.
18. **Finlay, C. A., P. W. Hinds, and A. J. Levine.** 1989. The p53 proto-oncogene can act as a suppressor of transformation. Cell **57**:1083–1093.
19. **Friedman, P. N., S. E. Kern, B. Vogelstein, and C. Prives.** 1990. Wild-type, but not mutant, human p53 proteins inhibit the replication activities of simian virus 40 large tumor antigen. Proc. Natl. Acad. Sci. USA **87**:9275–9279.
20. **Gannon, J. V., and D. P. Lane.** 1987. p53 and DNA polymerase α compete for binding to T antigen. Nature (London) **329**:456–458.
21. **Ginsberg, D., F. Mechta, M. Yaniv, and M. Oren.** 1991. Wild-type p53 can down-modulate the activity of various promoters. Proc. Natl. Acad. Sci. USA **88**:9979–9983.
22. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
23. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
24. **Hinds, P., C. Finlay, and A. J. Levine.** 1989. Mutation is required to activate the p53 gene for cooperation with the *ras* oncogene and transformation. J. Virol. **63**:739–746.
25. **Hinds, P. W., C. A. Finlay, R. S. Quartin, S. J. Baker, E. R. Fearon, B. Vogelstein, and A. J. Levine.** 1990. Mutant p53 DNA clones from human colon carcinomas cooperate with *ras* in transforming primary rat cells: a comparison of the "hot spot" mutant phenotypes. Cell Growth Differ. **1**:571–580.
26. **Hollstein, M., D. Sidransky, B. Vogelstein, and C. C. Harris.** 1991. p53 mutations in human cancers. Science **253**:49–53.
27. **Iggo, R., K. Gatter, J. Bartek, D. Lane, and A. L. Harris.** 1990.

Increased expression of mutant forms of p53 oncogene in primary lung cancer. Lancet 335:675–679.
28. **Jaskulski, D., C. Gatti, S. Travali, B. Calabretta, and R. Baserga.** 1988. Regulation of the proliferating cell nuclear antigen cyclin and thymidine kinase mRNA levels by growth factors. J. Biol. Chem. 263:10175–10179.
29. **Jenkins, J. R., K. Rudge, and G. A. Currie.** 1984. Cellular immortalization by a cDNA clone encoding the transformation associated phosphoprotein p53. Nature (London) 312:651–653.
30. **Kern, S. E., K. W. Kinzler, A. Bruskin, D. Jarosz, P. Friedman, C. Prives, and B. Vogelstein.** 1991. Identification of p53 as a sequence-specific DNA-binding protein. Science 252:1708–1711.
31. **Lane, D. P., and L. W. Crawford.** 1979. T antigen is bound to a host protein in SV40-transformed cells. Nature (London) 278: 261–263.
32. **Levine, A. J., J. Momand, and C. A. Finlay.** 1991. The p53 tumor suppressor gene. Nature (London) 351:453–456.
33. **Linzer, D. I. H., and A. J. Levine.** 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40 transformed cells and uninfected embryonal carcinoma cells. Cell 17:43–52.
34. **Malkin, D., F. P. Li, L. C. Strong, J. F. Fraumeni, C. E. Nelson, D. H. Kim, J. Kassel, M. Gryka, F. Z. Bischoff, M. A. Tainsky, and S. H. Friend.** 1990. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas and other neoplasms. Science 250:1233–1238.
35. **Maltzman, W., M. Oren, and A. J. Levine.** 1981. The structural relationships between 54,000 molecular weight cellular tumor antigens detected in viral and nonviral transformed cells. Virology 112:145–156.
36. **Martinez, J., I. Georgoff, J. Martinez, and A. J. Levine.** 1991. Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. Genes Dev. 5:151–159.
37. **McKnight, S. L., and R. Kingsbury.** 1982. Transcriptional control signals of a eukaryotic protein-coding gene. Science 217:316–324.
38. **Mercer, W. E., M. Amin, G. J. Sauve, E. Appella, S. J. Ullrich, and J. W. Romano.** 1990. Wild-type human p53 is antiproliferative in SV40-transformed hamster cells. Oncogene 5:973–980.
39. **Mercer, W. E., M. T. Shields, D. Lin, E. Appella, and S. J. Ullrich.** 1991. Growth suppression induced by wild-type p53 protein is accompanied by selective down-regulation of proliferating-cell nuclear antigen expression. Proc. Natl. Acad. Sci. USA 88:1958–1962.
40. **Michalivitz, D., O. Halevy, and M. Oren.** 1990. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. Cell 62:671–680.
41. **Morris, G. F., and M. B. Mathews.** 1990. Analysis of the proliferating cell nuclear antigen promoter and its response to adenovirus early region 1. J. Biol. Chem. 265:16116–16125.
42. **Mudryj, M., S. H. Devoto, S. W. Hiebert, T. Hunter, J. Pines, and J. R. Nevins.** 1991. Cell cycle regulation of the E2F transcription factor involves an interaction with cyclin A. Cell 65:1243–1253.
43. **Nabel, G. J., S. A. Rice, D. M. Knipe, and D. Baltimore.** 1987. Alternative mechanisms for activation of human immunodeficiency virus enhancer in T cells. Science 239:1299–1302.
44. **Nigro, J. M., S. J. Baker, A. C. Preisinger, J. M. Jessup, R. Hostetter, K. Cleary, S. H. Bigner, N. Davidson, S. Baylin, P. Devillee, T. Glover, F. S. Collins, A. Weston, R. Modali, C. C. Harris, and B. Vogelstein.** 1989. Mutations in the p53 gene occur in diverse human tumor types. Nature (London) 342:705–708.
45. **Parada, L. F., H. Land, R. A. Weinberg, D. Wolf, and V. Rotter.** 1984. Cooperation between gene encoding p53 tumour antigen and *ras* in cellular transformation. Nature (London) 312:649–651.
46. **Prelich, G., C. K. Tan, M. Kostura, M. B. Mathews, A. G. So, K. M. Downey, and B. Stillman.** 1987. Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxiliary protein. Nature (London) 326:517–520.
47. **Raycroft, L., H. Wu, and G. Lozano.** 1990. Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. Science 249:1049–1051.
48. **Rotter, V., M. A. Boss, and D. Baltimore.** 1981. Increased concentrations of an apparently identical cellular protein in cells transformed by either Abelson murine leukemia virus or other transforming agents. J. Virol. 38:336–346.
49. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1990. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
50. **Santhanam, V., A. Ray, and P. Sehgal.** 1991. Repression of the interleukin 6 gene promoter by p53 and the retinoblastoma susceptibility gene product. Proc. Natl. Acad. Sci. USA 88: 7605–7609.
51. **Sarnow, P., Y. Ho, J. Williams, and A. J. Levine.** 1982. The adenovirus E1b-58K tumor antigen and SV40 large tumor antigen are physically associated with the same 54K cellular protein. Cell 28:387–394.
52. **Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63:1129–1136.
53. **Sodroski, J. G., C. A. Rosen, and W. A. Haseltine.** 1984. *Trans*-acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. Science 225:381–385.
54. **Soussi, T., C. C. de Fromentel, and P. May.** 1990. Structural aspects of the p53 protein in relation to gene evolution. Oncogene 5:945–952.
55. **Stillman, B.** 1989. Initiation of eukaryotic DNA replication *in vitro*. Annu. Rev. Cell Biol. 5:197–245.
56. **Takahashi, T., M. M. Nau, I. Chiba, M. J. Birrer, R. K. Rosenberg, M. Vinocour, M. Levitt, H. Pass, A. F. Gazdar, and J. D. Minna.** 1989. p53: a frequent target for genetic abnormalities in lung cancer. Science 246:491–494.
57. **Thomas, R., L. Kaplan, N. Reich, D. P. Lane, and A. J. Levine.** 1983. Characterization of human p53 antigen employing primate specific monoclonal antibodies. Virology 131:502–517.
58. **Vogelstein, B.** 1990. Cancer: a deadly inheritance. Nature (London) 348:681–682.
59. **Wang, E. H., P. N. Friedman, and C. Prives.** 1989. The murine p53 protein blocks replication of SV40 DNA *in vitro* by inhibiting the initiation functions of SV40 large T antigen. Cell 57:379–392.
60. **Werness, B. A., A. J. Levine, and P. M. Howley.** 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science 248:76–79.

Vild-type mouse p53 down-regulates transcription from different virus nhancer/promoters

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he protein encoded by the tumour-suppressor gene p53 an complex with SV40 virus large T antigen, the denovirus E1B 58-kDa protein and the E6 protein of uman papillomavirus type 16. The functions of these omplexes are unclear, but there is some evidence to uggest that binding of p53 to these viral proteins may nactivate p53 function. Recent reports have shown that 53 is involved in regulation of transcription. We have onsidered the possibility that p53 may regulate trans-ription of viral genes important for virus replication nd/or transformation. Inactivation of p53 function by ormation of such complexes might then permit correct xpression of these viral genes. Since p53 can bind to the V40 virus enhancer/promoter, we have investigated the ffect of p53 on transcription from this promoter and eport here that mouse p53 is a potent repressor of the V40 enhancer/promoter. Mutations within p53 severely inhibited this activity and provided some evidence to show that the N-terminus of p53 contains residues essential for this function. We also show that mouse p53 represses transcription from the promoters of viruses that do not express proteins that complex with p53: the human cytomegalovirus early promoter and the Rous sarcoma virus long terminal repeat. By studying the effect of p53 on transcription in different cell lines, we show that the effects of p53 on promoters may be cell type specific.

Introduction

There is now considerable evidence to suggest that the nuclear phosphoprotein p53, in its wild-type form (wt), acts as a tumour suppressor. For example, deletions and mutations of the p53 gene are associated with a wide variety of human tumours (Masuda *et al.*, 1987; Baker *et al.*, 1989; Nigro *et al.*, 1989; Sidransky *et al.*, 1991; Takahashi *et al.*, 1991). In addition, wt p53 is able to reduce the tumorigenicity of transformed cells (Chen *et al.*, 1991; Cheng *et al.*, 1992) and suppress the transformation of primary rat cells by several combinations of transforming genes, including mutant p53 and the *ras* oncogene (Finlay *et al.*, 1989). Consistent with this role as a tumour suppressor, wt p53 has been shown to inhibit proliferation of tumour cells (Baker *et al.*, 1990; Diller *et al.*, 1990; Mercer *et al.*, 1990; Michalovitz *et al.*, 1990; Casey *et al.*, 1991; Isaacs *et al.*, 1991). Interestingly, wt p53 is also able to inhibit replication of SV40 virus DNA *in vitro* and *in vivo* (Braithwaite *et al.*, 1987; Sturzbecher *et al.*, 1988; Wang *et al.*, 1989).

The precise biochemical function of p53 is not yet clear. However, recent reports have provided evidence to suggest that at least one function of p53 may be in the regulation of transcription. Thus, studies in which the amino-terminal domain of p53 or intact p53 has been fused to the DNA-binding domain of the yeast GAL4 transcription factor have shown that p53 is able to stimulate transcription from a reporter gene containing multiple copies of the GAL4 DNA binding sites (Fields & Jang, 1990; O'Rourke *et al.*, 1990; Raycroft *et al.*, 1990). This activity is dramatically reduced when wt p53 sequences are replaced by p53 mutants (Raycroft *et al.*, 1990; 1991; Unger *et al.*, 1992). Mouse p53 is also able to activate transcription from the mouse muscle-specific creatine kinase (MCK) gene promoter (Weintraub *et al.*, 1991). In contrast to these observations, a number of studies have clearly shown that wt p53 can down-regulate expression from the retinoblastoma gene promoter (Shiio *et al.*, 1992) and from the promoters of genes whose expression is controlled by growth factors, such as interleukin 6 (Santhanam *et al.*, 1991), c-*fos*, c-*jun* and β-actin (Ginsberg *et al.*, 1991). In addition, expression of wt p53 reduces levels of proliferating cell nuclear antigen (PCNA) mRNA and protein (Mercer *et al.*, 1991). Thus p53 appears to be both a positive and negative regulator of transcription.

One of the best-characterized features of p53 is its ability to interact with viral proteins. p53 has been shown to form complexes with the SV40 large T antigen (Lane & Crawford, 1979; Linzer & Levine, 1979), adenovirus type 5 E1B 58-kDa antigen in both transformed cells (Sarnow *et al.*, 1982; Zantema *et al.*, 1985) and infected cells (Braithwaite *et al.*, 1991a) and the E6 protein of human papillomavirus type 16 (HPV-16) (Werness *et al.*, 1990). The functional significance of these interactions is unclear, but there is some evidence to suggest that binding of the viral proteins to p53 may inactivate p53 function. For example, binding of p53 to the E6 protein of HPV-16 causes a rapid degradation of p53 (Scheffner *et al.*, 1990).

Since p53 appears normally to function as a transcriptional regulator, we have explored the possibility that one of the functions for the complexes formed between p53 and viral proteins may be to prevent p53 affecting key viral and cellular genes that are essential to viral replication and/or transformation. Consistent with this idea, the adenovirus E1B protein is able to

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prevent transcriptional activation by p53 (Yew & Berk, 1992).

Recent data have shown that p53 can bind to the SV40 virus enhancer/promoter (Bargonetti *et al.*, 1991). Therefore, we examined the effect of p53 on transcription driven from the SV40 enhancer/promoter using a transient expression system in which a reporter plasmid containing the bacterial chloramphenicol acetyl transferase (CAT) gene fused downstream of the SV40 enhancer/promoter (pSV2CAT) was co-transfected with vectors expressing mouse p53.

Under these conditions, we show that wt mouse p53 is a potent down-regulator of the SV40 enhancer/promoter and also of other viral enhancer/promoters. Mutations within p53 severely inhibited this down-regulation. In addition, using data from studies with our p53 mutants, we provide preliminary evidence that the N-terminal domain of p53 is essential for down-regulation. We also show that the effects of p53 on certain promoters may be cell type specific.

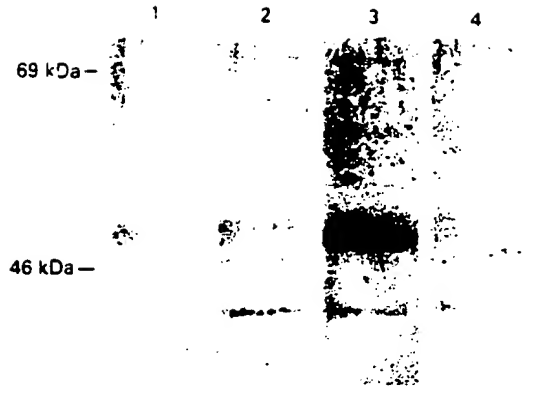

Figure 1 Expression of wt mouse p53 in transfected HeLa cells. HeLa cells were transfected with 20 μg of control plasmid (lanes 1 and 2) or 10 μg each of control plasmid and pCMVNc9 (lanes 3 and 4). After 72 h, cells were labelled for a further 2 h with [^{35}S]methionine and cell lysates prepared. Lysates were then immunoprecipitated with either normal mouse serum (lanes 2 and 4) or PAb 122, a mouse-specific monoclonal antibody (lanes 1 and 3), as described in Materials and methods. Positions of molecular weight markers are indicated

Results

Specific down-regulation of transcription from the SV40 enhancer/promoter by mouse p53

A number of reports have investigated the effect of p53 on transcription using a 'fusion protein' approach in which fragments of p53 are fused to the DNA-binding domain of GAL4 yeast transcription factor (see Introduction). In particular, Raycroft *et al.* (1990) showed that a GAL4–p53 fusion protein is able to activate transcription from a reporter plasmid containing multiple copies of the GAL4 DNA-binding sequence upstream of the SV40 promoter. We have been concerned that the data obtained from such 'fusion protein' approaches has not always reflected the true biological function of proteins (see for example Braithwaite *et al.*, 1991b). Therefore, to overcome any potential problems, we have investigated the effect of p53 on transcription from the SV40 enhancer/promoter without GAL4 binding sites in a transient transfection system. In this system, the reporter plasmid contained the SV40 enhancer/promoter inserted upstream of the bacterial CAT gene (pSV2CAT; Gorman *et al.*, 1982a). This plasmid was introduced into human HeLa cells, along with equal amounts of a plasmid expressing wt mouse p53 from the human cytomegalovirus (CMV) immediate-early promoter (pCMVNc9; Eliyahu *et al.*, 1989). HeLa cells were chosen because they do not express endogenous p53 protein (Benchimol *et al.*, 1982), which might complicate interpretation of results.

The absence of endogenous p53 protein in HeLa cells is probably due to the expression of E6 protein from human papillomavirus type 18 in these cells (Banks *et al.*, 1987), which is able to complex with and degrade human p53 protein (Scheffner *et al.*, 1990). It is possible that in our studies the introduced mouse p53 might also be degraded. Therefore, in an initial experiment, we confirmed expression of intact mouse p53 in HeLa cells after transfection, by labelling cells with [^{35}S]methionine and immunoprecipitation of p53 from cells lysates using PAb 122 (Gurney *et al.*, 1980), a mouse p53 monoclonal antibody (Figure 1).

We next examined the effect of wt mouse p53 on the SV40 enhancer/promoter. Three days after co-transfection of pSV2CAT with either a control plasmid (p19Kan; Pridmore, 1987) or pCMVNc9, cell extracts were prepared and assayed for CAT activity by the method of Sleigh (1986). Results presented in Figure 2a indicate that CAT activity driven from the SV40 enhancer/promoter was more than 50-fold lower in cells co-transfected with pCMVNc9 than in cells transfected with the control. This effect of p53 was shown to be specific by replacing pCMVNc9 with either pCMVR (a plasmid containing the CMV promoter but encoding no protein) or pCMVFraCOOH (expressing residues 136–275 of the Fra-1 transcription factor, a protein unrelated to p53; Cohen *et al.*, 1989). There was no significant effect on CAT expression from the SV40 promoter/enhancer in cells co-transfected with either pCMVR or pCMVFraCOOH (Figure 2a).

By varying the amounts of pCMVNc9, we found a clear dose-dependent decline in SV40 enhancer/promoter activity with increasing amounts of plasmid expressing wt p53 (Figure 2b). A 50% inhibition of CAT activity was observed at approximately 0.75 μg of pCMVNc9 plasmid DNA.

In a separate set of experiments, we obtained similar, specific down-regulation of the SV40 enhancer/promoter in mouse L929 cells (fivefold reduction) and monkey CV1 cells (20-fold reduction), even though both cell lines express endogenous p53 (data not shown).

Kinetics of transcriptional down-regulation by p53

Overexpression of wt p53 has been shown to arrest cellular growth at a point near the G_1/S phase boundary of the cell cycle (Diller *et al.*, 1990; Mercer *et al.*, 1990; Michalovitz *et al.*, 1990; Martinez *et al.*, 1991). Even though we were assaying CAT activity in lysates derived from equal numbers of cells, we were concerned that the effect of p53 on expression from the SV40 enhancer/promoter might be due to a secondary effect of slowed cell growth. Therefore, we performed a

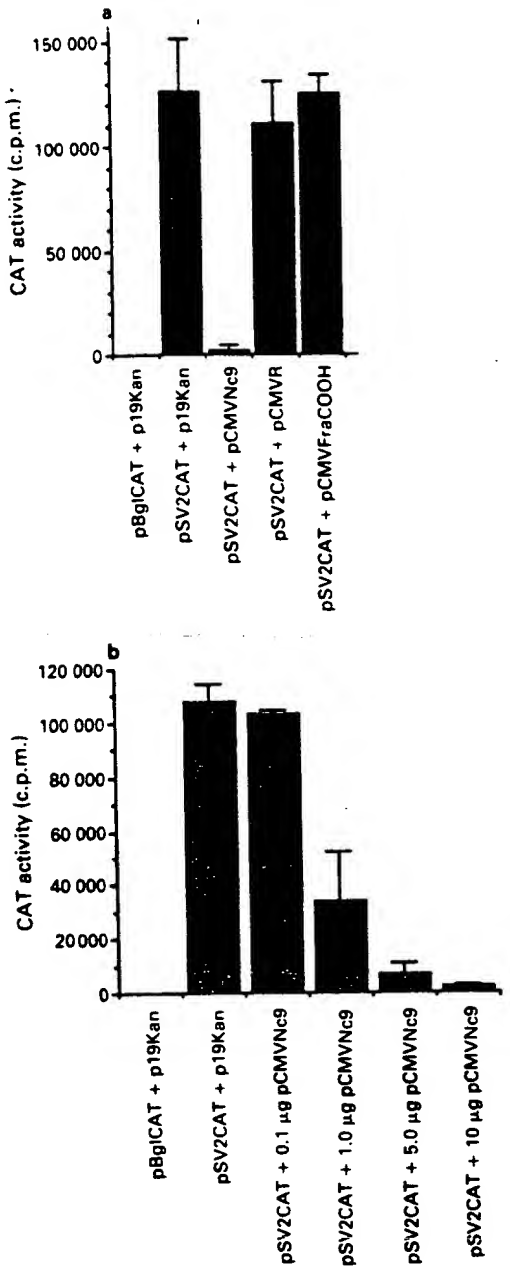

Figure 2 Specific down-regulation of the SV40 enhancer/promoter by mouse p53. (**a**) HeLa cells were co-transfected with 10 μg each of the indicated plasmids, as described in Materials and methods. pBglCAT is a control plasmid that lacks the SV40 enhancer/promoter sequences upstream of the CAT gene (Gorman *et al.*, 1982a). p19Kan is a non-specific control plasmid (Pridmore, 1987). After 72 h, cell lysates were prepared, normalized for protein content (hence cell number) and used to determine CAT activity as described in Materials and methods. Data are presented as the means and standard deviations of three independent transfections, each assayed in duplicate. (**b**) HeLa cells were transfected with 10 μg of CAT reporter plasmid and increasing amounts of pCMVNc9 as indicated. In all cases, the total amount of DNA transfected was maintained at 20 μg by the addition of control plasmid. Data presented are the means with standard deviations of two independent transfections, each assayed in duplicate

time-course experiment, in which cells were co-transfected with pSV2CAT and either a control plasmid or pCMVNc9. The results presented in Figure 3 show that in cells co-transfected with pSV2CAT and the control plasmid no CAT activity was detected 8 h after transfection. However, low levels of CAT activity were detected by 24 h after transfection, which increased to a maximum after 72 h. In cells transfected with pSV2CAT and pCMVNc9, inhibition of the low levels of CAT activity observed 24 h after transfection (97%) was as prominent as the inhibition of the high levels of CAT activity observed 72 h after transfection. At this time (24 h), we found no difference in cell number between samples containing control plasmid or pCMVNc9 (data not shown). Although not conclusive evidence, these results suggest that the effect of p53 on the SV40 enhancer/promoter is not due to slowed cell growth.

p53 mutants have reduced ability to down-regulate transcription

Recent reports have indicated that many p53 mutants have impaired ability to regulate either transcriptional activation (Raycroft *et al.*, 1990; 1991; Weintraub *et al.*, 1991; Kern *et al.*, 1992; Unger *et al.*, 1992) or repression (Santhanam *et al.*, 1991). We wanted to determine the effect of mutations within p53 on the ability of wt p53 to down-regulate transcription from the SV40 enhancer/promoter. Therefore, a number of plasmids that express mutant p53 proteins (Figure 4a) were co-transfected into HeLa cells along with pSV2CAT. The results presented in Figure 4b show that only pCMVNc9 and pCMVmsp53 were able to significantly reduce transcription from this promoter when compared with the control. The msp53 was, however, much less effective than wt p53, reducing activity only about fivefold. The ability of all other mutants, pCMVdl 163, pCMVdl518 and pCMVc5, was abrogated.

To exclude the possibility that failure to down-regulate transcription was due to a failure of p53 expression in the transfected cells, HeLa cells were separately transfected with all the p53 constructs and labelled with [^{35}S]methionine. Cell lysates were then immunoprecipitated with a monoclonal antibody specific for p53, PAb 122 (Gurney *et al.*, 1980). The results (Figure 4c) show that all constructs expressed a p53 protein of the expected size and that mutant proteins from pCMVdl 163, pCMVdl 518, and pCMVc5 were all expressed at levels equivalent to or greater than pCMVNc9 (lanes 2, 3, 4 and 6). Thus, loss of down-regulation of pSV2CAT by these mutant p53 proteins cannot be explained by failure to express p53. Mutant msp53, however, was expressed at a much lower level than pCMVNc9 (lanes 2 and 5), suggesting that the reduced ability of this mutant to down-regulate transcription may at least in part be due to the reduced levels of protein expression.

wt p53 modulates the activity of other viral promoters

p53 is able to complex with SV40 large T antigen, and we have shown in this report that mouse p53 is able to down-regulate expression from the SV40 enhancer/promoter. However, we were also interested to determine whether p53 is able to affect transcription from the promoters of viruses that do not express proteins that bind to p53. Therefore, reporter plasmids in which the bacterial CAT gene is fused upstream of the human

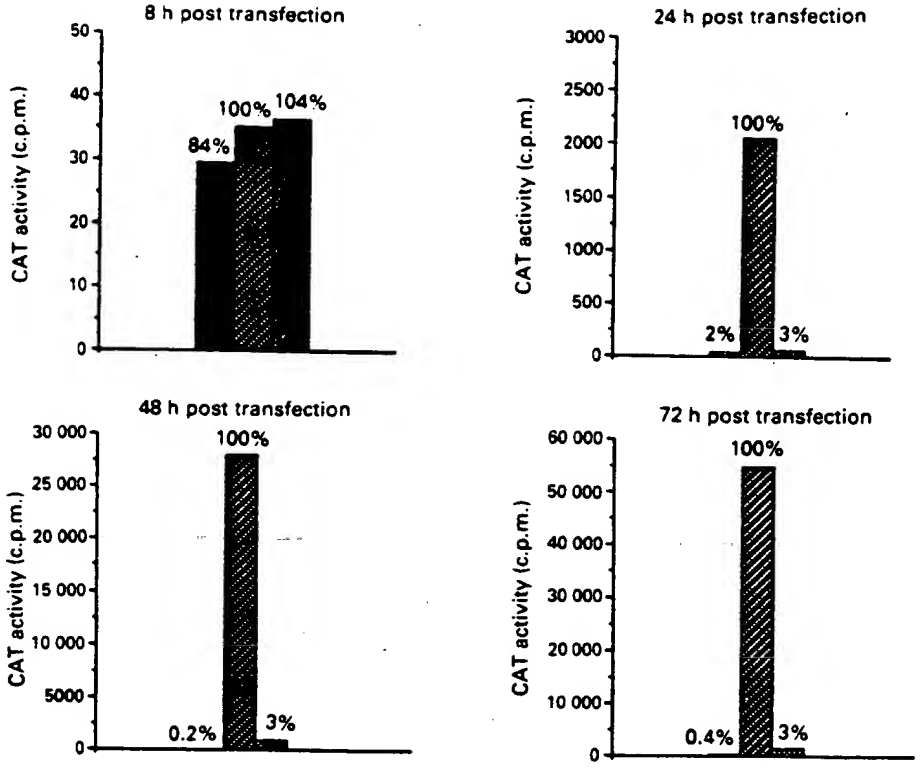

Figure 3 Kinetics of transcriptional repression by p53. HeLa cells were co-transfected with 10 μg of pBglCAT and 10 μg of control plasmid, p19Kan (black bar), or 10 μg of pSV2CAT with either 10 μg of control plasmid (hatched bar) or pCMVNc9 (stippled bar). At the indicated times after addition of DNA, cells were harvested and assayed for CAT activity as described in Materials and methods

cytomegalovirus immediate-early promoter (pRcCMV-CAT) or the Rous sarcoma virus long terminal repeat (RSV-LTR) (pRSVCAT; Gorman *et al.*, 1982b) were co-transfected into HeLa cells with pCMVNc9. The data presented in Table 1 show that expression of p53 resulted in down-regulation of CAT expression from both the CMV promoter (twofold) and RSV-LTR (16-fold), though the levels of down-regulation were less than those observed for the SV40 enhancer/promoter. In addition, to ensure that the effects seen with p53 on the CMV and RSV promoters were not due to competition between promoters for limiting transcription factors, we replaced the control plasmid, p19Kan, with the plasmid pCMVR. We observed no reduction in activity obtained from these promoters (data not shown). These results demonstrate that down-regulation by p53 is not unique to the SV40 virus enhancer/promoter.

As a control for the effects of p53 expressed from pCMVNc9 on the viral promoters used in our system, we decided to analyse the effect of this p53 on the mouse muscle-specific creatine kinase (MCK) gene promoter. In monkey CV1 cells, this promoter has been shown to be activated by mouse p53 (Weintraub *et al.*, 1991). More specifically, by using increasing amounts of MCK promoter sequence, this group showed that p53 is able to activate the MCK promoter 10- to 80-fold when a 500-bp fragment containing the putative 'p53-responsive element' is present, but some activation (two- to fivefold) was also observed in the absence of this 500-bp sequence. The following MCK gene promoter–CAT constructs (Weintraub *et al.*, 1991) were co-transfected into human HeLa cells, mouse L929 cells or monkey CV1 cells along with equal amounts of either a control plasmid or pCMVNc9: p3300MCK-CAT (containing promoter sequences from −3300 to +7), p2800MCKCAT (containing promoter sequences from −2800 to +7 and lacking the p53-responsive element) and p80MCKCAT (containing promoter sequences from −80 to +7). The results presented in Table 2 show that in CV1 cells we obtained levels of activation from the MCK promoter by mouse p53 in the absence (sixfold) and presence (20-fold) of the putative p53-responsive element that are similar to those observed by Weintraub *et al.* (1991). Interestingly, in HeLa cells and L cells, in which the levels of expressed p53 were much higher than that observed in CV1 cells (data not shown), we found that p53 caused no activation of the MCK promoter even when the p53-responsive element was present. Thus, some of the effects of p53 appear to be cell type specific. In addition, we consistently found that in all three cell types tested, but most clearly in CV1 and HeLa cells, p53 down-regulated the low levels of activity we obtained from the p80MCKCAT plasmid.

Discussion

In this report, we have shown that wt mouse p53 is able specifically to down-regulate expression from the SV40 enhancer/promoter and the promoters of other viruses. p53 can also suppress transcription from the promoters of several growth factor-responsive genes

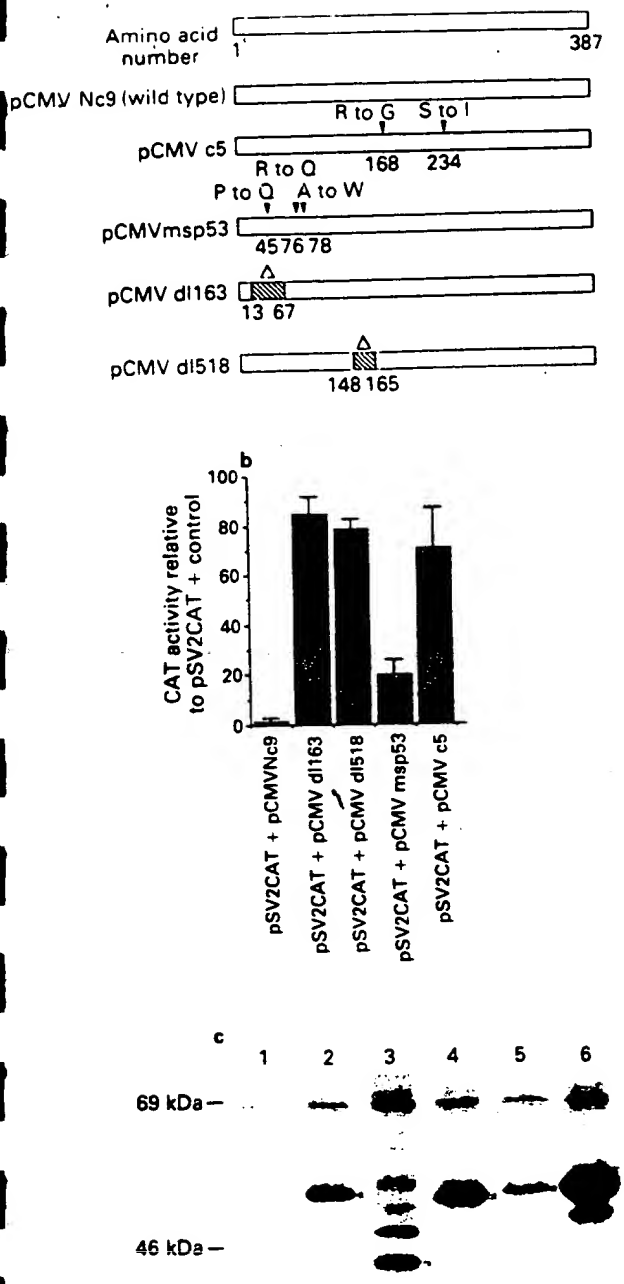

Figure 4 Effect of deletions and point mutations in p53 on transcription from pSV2CAT. (a) Schematic illustration of p53 mutants used. Hatched areas indicate deletions. Abbreviations for amino acid residues are: A, Ala; G, Gly; I, Ile; P, Pro; Q, Gln; R, Arg; S, Ser; and W, Trp. p53 mutants dl 163, dl 518, msp53 and c5 were all expressed from the human CMV enhancer/promoter in plasmids pCMVdl163, pCMVdl518, pCMVmsp53 (Jenkins *et al.*, 1985; Braithwaite *et al.*, 1987; Sturzbecher *et al.*, 1988) and pCMVc5 (Eliyahu *et al.*, 1989) respectively. (b) HeLa cells were co-transfected with the 10 μg of CAT reporter plasmid and 10 μg of either control plasmid or plasmid expressing p53, and assayed for CAT activity as described in Materials and methods. Results presented are the means with standard deviations of three independent transfections, each assayed in duplicate. (c) Expression of wild-type and mutant p53 proteins in transfected cells. HeLa cells were transfected with a control plasmid (lane 1) or plasmids expressing wt p53 (pCMVNc9) (lane 2), dl 163 (pCMVdl163) (lane 3), dl 518 (pCMVdl518) (lane 4), msp53 (pCMVmsp53) (lane 5) or c5 (pCMVc5) (lane 6). After 72 h, transfected cells were labelled with [^{35}S]methionine for a further 2 h; cell lysates were then prepared and immunoprecipitated with PAb 122, a mouse p53 monoclonal antibody, as described Materials and methods. Asterisks indicate the precipitated p53 proteins. Positions of molecular weight markers are indicated

Table 1 Regulation of transcriptional activity from different promoters by wt mouse p53

	*Relative CAT activity**		
	Plasmids co-transfected with CAT reporter		
CAT reporter plasmid	*p19Kan (control)*	*pCMVNc9 (wt p53)*	*Effect of p53*
pSV2CAT	100	1.1 ± 0.04	91-fold reduction
pRSVCAT	100	6.1 ± 5.8	16-fold reduction
pRcCMVCAT	100	32.5 ± 17.6	2-fold reduction

*HeLa cells were transfected with 10 μg of each of the indicated plasmids and assayed for CAT activity, as described in Materials and methods. In each case, levels of CAT activity obtained from cells transfected with pRSVCAT and pRcCMVCAT were similar to those obtained with pSV2CAT. Results presented are the means and standard deviations of three independent transfections, each assayed in duplicate

Table 2 Modulation of transcriptional activity from the mouse MCK gene promoter by wt mouse p53

	*Relative CAT activity**		
	Plasmids co-transfected with CAT reporter		
CAT reporter plasmid	*p19Kan (control)*	*pCMVNc9 (wt p53)*	*Effect of p53*
CV1 cells			
p80MCKCAT	1.00	0.2 ± 0.2	5-fold reduction
p2800MCKCAT	1.00	6.3 ± 1.6	6-fold activation
p3300MCKCAT	1.00	19.3 ± 0.8	19-fold activation
HeLa cells			
p80MCKCAT	1.00	0.1 ± 0.1	10-fold reduction
p2800MCKCAT	1.00	0.8 ± 0.6	No effect
p3300MCKCAT	1.00	1.6 ± 1.1	No effect
L929 cells			
p80MCKCAT	1.00	0.5 ± 0.3	No effect
p2800MCKCAT	1.00	1.4 ± 0.6	No effect
p3300MCKCAT	1.00	2.8 ± 2.2	3-fold activation

*Cells were transfected with 10 μg of each of the indicated plasmids and assayed for CAT activity, as described in Materials and methods. Results presented are the means with standard deviations of two (CV1), three (L cells) and four (HeLa) independent transfections, each assayed in duplicate

(Ginsberg *et al.*, 1991; Santhanam *et al.*, 1991), the retinoblastoma gene (Shiio *et al.*, 1992) and the multidrug resistance gene (Chin *et al.*, 1992). The mechanism by which p53 down-regulates transcription is not clear. Recently, however, Shiio *et al.* (1992) identified a short, p53-responsive element in the retinoblastoma (Rb) gene promoter. This G(G/C)AA(G/C)TGA motif was required for specific down-regulation of the Rb promoter by p53. A similar sequence (GGAACTGG) is present in the SV40 enhancer/promoter (+57 to +64 of SV40 DNA) and is the centre of a domain to which both human and mouse p53 binds (Bargonetti *et al.*, 1991). Although we have no direct evidence, we would suggest that transcription from the SV40 enhancer/promoter is down-regulated by mouse p53 through binding to this octamer sequence.

Binding of p53 to a specific DNA sequence may be required for down-regulation of certain promoters, but there is also evidence to suggest that other mechanisms of down-regulation may be involved. Thus, p53 cannot bind DNA sequences from the promoters of growth factor-responsive genes (Santhanam *et al.*, 1991), and the p53-responsive element identified by Shiio *et al.*

(1992) is not present in the CMV and RSV-LTR promoters used in this study. It is also absent from both the published rat and human PCNA gene promoters (Travali *et al.*, 1989; Ohashi *et al.*, 1992) even though mouse p53 is able to down-regulate expression from the PCNA promoter (P. Jackson, unpublished data). In the absence of DNA binding, the mechanism of transcriptional repression is unclear. However, it is possible that p53 may act by interacting with one or more key transcription factors to prevent their binding to regulatory sequences.

p53 has been shown to activate transcription from both artificial promoters (containing elements of the SV40 promoter and the adenovirus E1B TATA box) (Fields & Jang, 1990; O'Rourke *et al.*, 1990; Raycroft *et al.*, 1990) and natural promoters (Weintraub *et al.*, 1991). Recently, DNA footprinting has revealed that human p53 can bind two fragments of DNA containing a short, tandemly repeated sequence (Kern *et al.*, 1991). When placed upstream of an artificial promoter, the sequence enabled p53-specific activation of that promoter (Kern *et al.*, 1992). This p53-responsive element is also present in the mouse muscle-specific creatine kinase gene promoter (MCK) and allows p53 activation from this promoter (Weintraub *et al.*, 1991; this manuscript, Table 2). Interestingly, within each of the p53-binding fragments identified by Kern *et al.* (1991) are motifs similar to the consensus sequence identified by Shiio *et al.* (1992): GGAACTGT and GCAAGTC. The mechanism by which p53 can both activate and repress transcription from apparently similar sequences is not clear. Conceivably, p53 may act with similar factors for both activation and repression. Consistent with this notion, the amino-terminal domain of p53 appears essential for both transcriptional activation and repression. For activation, binding of p53 to the DNA through its specific element and to a particular factor(s) could promote binding of the transcription factor(s) to its regulatory element and/or alter the DNA conformation. In either case, the result is to activate a quiescent promoter. In contrast, in an active promoter, binding of p53 to its specific sequence may prevent binding of similar transcription factor(s), thereby inhibiting transcription.

In an initial series of experiments, Weintraub *et al.* (1991) showed that, in all cell types tested, wt mouse p53 was able to activate transcription from the mouse MCK promoter. Thus, p53 was able to activate the MCK promoter by similar amounts in monkey CV1 cells, human HepG2 cells and mouse CH3/10T½ cells. In contrast, in our experiments with CV1 cells, mouse L929 and human HeLa cells the effects of p53 show some cell type specificity, since p53 was unable to significantly activate transcription from the MCK promoter in either L cells or HeLa cells. These data would suggest that factors involved in transcriptional activation by p53 are also cell type specific, thus depending on the context p53 may or may not activate particular genes. The effects of p53 may also be species specific, since human wt p53 is unable to activate the MCK promoter in CV1 cells (Weintraub *et al.*, 1991) or down-regulate the SV40 enhancer/promoter in HeLa cells (Shiio *et al.*, 1992). However, after having prepared this manuscript, Subler *et al.* (1992) reported that human wt p53 can down-regulate transcription from a number of viral and cellular promoters including the SV40 enhancer/promoter, CMV enhancer promoter and the RSV-LTR in NIH3T3 and VERO cells. Again, these data would argue that cell-specific factors are important for p53 function.

A number of recent reports have shown that many p53 mutants have impaired ability to regulate either transcriptional activation (Raycroft *et al.*, 1990; 1991; Weintraub *et al.*, 1991; Kern *et al.*, 1992; Unger *et al.*, 1992) or repression (Santhanam *et al.*, 1991). Consistent with these observations, we have shown that mutations in most cases abrogate the ability of p53 to down-regulate transcription from the SV40 enhancer promoter (Figure 4a and b). The loss of function observed for the mutants used in this study was not due to a failure to express the particular mutant protein (Figure 4c), though the reduced activity observed for pCMVmsp53 may well be explained by the fact that it was expressed at much lower levels than the wt p53.

The fact that the p53 mutants pCMVc5 and pCMVdl 518 have severely reduced ability to down-regulate the SV40 enhancer/promoter might suggest that the sequences within these p53 proteins affected by their mutations (both in the central region of the p53 protein) are important for the transcriptional down-regulation function of p53. However, it is also possible that these mutations alter the structure of the p53 protein and affect the structure of the true functional domain, which lies elsewhere on the protein. Consistent with this idea, these two mutant proteins fail to react with the conformation-sensitive monoclonal antibody PAb 246, whose epitope lies in the amino-terminal region of p53 (Jenkins & Sturzbecher, 1988). Rather than identifying a possible functional domain in p53, the simplest interpretation of the data for pCMVc5 and pCMVdl518 is that the mutants have adopted an overall unfavourable conformation for down-regulation. On the other hand, pCMVdl163 is in the wt conformation, as determined by its immunoreactivity with PAb 246 (Sturzbecher *et al.*, 1988), so the effect of the mutation in this p53 can be more clearly evaluated. pCMVdl163 almost fails to down-regulate the SV40 enhancer/promoter. These data suggest that residues 13–67 are important for the ability of mouse p53 to down-regulate the SV40 enhancer/promoter. Similar results for pCMVdl163 were obtained in experiments with the CMV enhancer/promoter and the RSV-LTR (P. Jackson, unpublished data). Although a more extensive series of mutants needs to be investigated, our preliminary conclusion is that an N-terminal domain of p53 may contain sequences responsible for down-regulation of the SV40 and other viral enhancer promoters. Interestingly, this domain appears to be essential for transcriptional activation by p53 (Fields & Jang, 1990; Unger *et al.*, 1992) and is also the domain to which the adenovirus E1B 58-kDa protein binds (Braithwaite *et al.*, 1991a).

Recently, Yew & Berk (1992) reported that the adenovirus E1B 58-kDa protein can suppress p53-mediated activation of both an artificial promoter and the MCK promoter in BRK cells. This finding is consistent with the possibility that at least one of the functions for the interaction of viral proteins with p53 may be to overcome transcriptional regulation by p53. Hence, it will be of interest to identify whether or not p53 can affect transcription from any adenovirus gen

romoter. Within the context of this report, it will also e of importance to determine whether or not E1B 5-kDa can overcome the p53-mediated down-regulation of the SV40 enhancer promoter.

Materials and methods

Cells

Human HeLa cells, mouse L929 cells and monkey CV1 cells were all routinely maintained in minimal essential medium (Gibco BRL, Grand Island, NY, USA) containing 0.22% sodium bicarbonate and 10% heat-inactivated fetal calf serum (FCS).

Plasmids

Plasmids pSV2CAT expressing the bacterial CAT gene from the SV40 early enhancer/promoter (Gorman *et al.*, 1982a) and pBglCAT, a promoterless CAT plasmid (previously called pSV0, Gorman *et al.*, 1982a), were both generous gifts from M. Sleigh (CSIRO, North Ryde, Australia).

Plasmid pCMVR, containing the human CMV immediate-early promoter but encoding no protein, and plasmid pCMVFra-COOH, encoding residues 136–275 of the Fra-1 protein expressed from the CMV immediate-early promoter (Cohen *et al.*, 1989), were gifts from D. Cohen (John Curtin School of Medical Research, Canberra, Australia).

Plasmids pCMVNc9 (Eliyahu *et al.*, 1989) encoding wild-type murine p53 and pCMVc5 (Eliyahu *et al.*, 1989) expressing murine p53 cDNA containing point mutations affecting amino acid residues 168 (R to G) and 234 (S to I) both expressed from the human CMV immediate-early promoter were obtained from M. Oren (Weizmann Institute, Rehovot, Israel).

Plasmid pRSVCAT expressing the CAT gene from the Rous sarcoma virus 3' LTR (Gorman *et al.*, 1982b) was obtained from B. van Leeuwen (John Curtin School of Medical Research, Australia).

The non-specific control plasmid p19Kan, a derivative of pUC19 in which the bacterial ampicillin resistance gene is replaced by a bacterial kanamycin resistance gene (Pridmore, 1987), was obtained from D. Pridmore, (Ciba-Geigy, Basle, Switzerland).

Plasmids encoding a mutant p53 lacking amino acid residues 148–165 (pCMVdl 518), lacking residues 13–67 (pCMVdl163) and with point mutations at residues 45 (R to Q), 76 (P to Q) and 78 (A to W) (pCMVmsp53JJ) (Jenkins *et al.*, 1985; Braithwaite *et al.*, 1987; Sturzbecher *et al.*, 1988), all expressed from the human CMV immediate-early promoter, were obtained from J. Jenkins (Marie Curie Research Institute, Oxted, UK).

Plasmid pRcCMVCAT, expressing the bacterial CAT gene from the human CMV immediate-early promoter, was obtained from R. Reddel (Children's Medical Research Foundation, Sydney, Australia).

Plasmids containing sequences from the mouse-specific creatine kinase (MCK) gene promoter fused to the bacterial CAT gene, p3300MCKCAT, p2800MCKCAT and p80-MCKCAT (Weintraub *et al.*, 1991), were gifts from S. Hauschka (University of Washington, Seattle, WA, USA).

All plasmid DNA preparations for transfection were purified by caesium chloride equilibrium density centrifugation essentially as described in Sambrook *et al.* (1989).

Transfection and assay of chloramphenicol acetyl transferase (CAT) activity

Cells were transfected with 10 μg of CAT reporter plasmid and 10 μg of either a control plasmid or p53 expression plasmid, essentially as described by Chen & Okayama (1987). Briefly, 5×10^5 cells were seeded into 10-cm Petri dishes and incubated overnight in 10 ml of growth medium. Plasmid DNA was then mixed with 0.5 ml of 0.25 M calcium chloride and 0.5 ml of 2 × BBS (50 mM BES pH 6.95, 280 mM sodium chloride, 1.5 mM disodium hydrogen phosphate) and the mixture incubated for 20 min at room temperature. The DNA solution was then added to the cells, dishes were gently swirled and incubated overnight in 3% carbon dioxide, at 35°C. The medium was removed, cells were washed twice in growth medium, refed and incubation continued for a further 48 h at 5% carbon dioxide, 37°C, until harvested.

The level of CAT activity in transfected cell lysates was determined essentially as described by Sleigh (1986). Each dish of transfected cells was washed twice in ice-cold phosphate-buffered saline (PBS). Cells were harvested by scraping into 1 ml of ice-cold PBS, pelleted and finally resuspended in 200 μl of 0.25 M Tris-Cl pH 7.8. Extracts of transfected cells were then prepared by three rounds of freezing (dry ice, 6 min) and thawing (37°C, 3 min). Cell suspensions were vortexed thoroughly before each freezing step. Cell debris was removed by centrifugation for 15 min at 12 000 *g* and 4°C. The supernatant was then heated to 65°C for 10 min to inactivate a CAT inhibitor previously identified by Sleigh (1986).

To enable addition of lysates from equal numbers of cells from different samples into the CAT assay, the protein content in each cell extract was estimated by measurement of the absorbance at 280 nm.

CAT activity in cell lysates was assayed by measuring the transfer of [^{14}C]acetyl groups from [^{14}C]acetyl CoA into chloramphenicol as follows. Reaction mixtures contained cell lysate from transfected cells (normally approximately 30 μl), 20 μl of 8 mM chloramphenicol, 20 μl of diluted [^{14}C]acetyl CoA (60 mCi mmol^{-1}, Amersham). Prior to use, 0.5 μCi (10 μl) of [^{14}C]acetyl CoA was diluted 10-fold with cold acetyl CoA (0.5 mM in 0.25 M Tris-Cl pH 7.5) and 0.25 M Tris-Cl pH 7.5 to a total volume of 100 μl. In each assay, volumes of cell lysate and 0.25 M Tris-Cl pH 7.5 were adjusted to account for differences in protein content so that the CAT activity was determined from equal numbers of cells from different samples. Reactions were allowed to proceed for 2 h at 37°C. Labelled products were then extracted into 2 × 10 μl of ice-cold ethyl acetate. Layers were vigorously mixed and separated by centrifugation at 12 000 *g* for 3 min. After each extraction, the organic phase containing the labelled products was removed. To ensure no transfer of labelled substrate into the organic phase, a final back-extraction was performed on the combined organic phase with 100 μl of 0.25 M Tris-Cl pH 7.5. After vigorous mixing and centrifugation at 12 000 *g*, 100 μl of the organic phase was placed into 5-ml polyethylene vials, 4 ml of ReadySafe Scintillation Cocktail was added (Beckman Instruments, CA, USA) and the radioactivity determined by scintillation counting. In all experiments, radioactivity was measured as counts per min (c.p.m.) obtained from 5 min of counting.

Immunoprecipitations

Petri dish cultures of transfected cells washed twice with PBS were incubated at 37°C, 5% carbon dioxide, in 2 ml of methionine-free minimal essential medium (Flow Laboratories, Irvine, UK) containing 1% L-glutamine and 2% FCS. After 30 min, the medium was removed, fresh methionine-free medium added and the incubation continued for a further 30 min. This medium was then removed, and the cells incubated in 2 ml of fresh methionine-free medium containing 100 μCi ml^{-1} ^{35}S-Translabel (1192 Ci mmol^{-1}, ICN Biomedicals, Irvine, CA, USA) for 2 h at 37°C, 5% carbon dioxide.

After removing the labelling medium and washing twice with ice-cold PBS, cells were lysed in 1 ml of RIPA buffer

(10 mM Tris-Cl pH 8.0, 150 mM sodium chloride, 1 mM EDTA, 1% NP-40, 0.1% SDS, 30 μg ml^{-1} aprotonin). After 20 min, lysates were precleared at 120 000 *g* for 20 min. Immunoprecipitations were then carried out using protein A-Sepharose as described in Zhang *et al.* (1990). Precipitated proteins were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography.

Antibodies

p53 proteins were immunoprecipitated from transfected cells with the monoclonal antibody PAb 122 (Gurney *et al.*, 1980). Normal mouse serum (NMS) was prepared from mice bred at the John Curtin School of Medical Research under specif pathogen-free conditions.

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References

Baker, S.J., Fearon, E.R., Nigro, J.M., Hamilton, S.R., Preisinger, A.C., Jessup, J.M., van Tuinen, P., Ledbetter, D.H., Barker, D.F., Nakamura, Y., White, R. & Vogelstein, B. (1989). *Science*, **244**, 217–221.

Baker, S.J., Markowitz, S., Fearon, E.R., Willson, J.K.V. & Vogelstein, B. (1990). *Science*, **249**, 912–915.

Banks, L., Spence, P., Androphy, E., Hubbert, N. Matlashewski, G., Murray, A. & Crawford, L. (1987). *J. Gen. Virol.*, **68**, 1351–1359.

Bargonetti, J., Friedman, P.N., Kern, S.E., Vogelstein, B. & Prives, C. (1991). *Cell*, **65**, 1083–1091.

Benchimol, S., Pim, D. & Crawford, L. (1982). *EMBO J.*, **1**, 1055–1062.

Braithwaite, A.W., Sturzbecher, H.-W., Addison, C., Palmer, C., Rudge, K. & Jenkins, J.J. (1987). *Nature*, **329**, 458–460.

Braithwaite, A.W., Blair, G.E., Nelson, C.C., McGovern, J. & Bellett, A.J.D. (1991a). *Oncogene*, **6**, 781–787.

Braithwaite, A.W., Nelson, C.C. & Bellett, A.J.D. (1991b). *New Biol.*, **3**, 18–26.

Casey, G., Lo-Hsueh, M., Lopez, M.E., Vogelstein, B. & Stansbridge, E.J. 1991). *Oncogene*, **6**, 1791–1797.

Chen, C. & Okayama, H. (1987). *Mol. Cell. Biol.*, **7**, 2745–2752.

Chen, Y., Chen, P.-L., Arnaiz, N., Goodrich, D. & Lee, W.-H. (1991). *Oncogene*, **6**, 1799–1805.

Cheng, J., Yee, J.-K., Yeargin, J., Friedmann, T. & Haas, M. (1992). *Cancer Res.*, **52**, 222–226.

Chin, K.-V., Ueda, K., Pastan, I. & Gotteman, M. (1992). *Science*, **255**, 459–462.

Cohen, D.R., Ferreira, P.C.P., Gentz, R., Franza Jr, B.R. & Curran, T. (1989). *Genes Dev.*, **3**, 173–184.

Diller, L. Kassel, J., Nelson, C., Gryka, M., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S., Vogelstein, B. & Friend, S. (1990). *Mol. Cell. Biol.*, **10**, 5772–5781.

Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kimhi, O. & Oren, M. (1989). *Proc. Natl. Acad. Sci. USA*, **86**, 8763–8767.

Fields, S. & Jang, S.K. (1990). *Science*, **249**, 1046–1049.

Finlay, C.A., Hinds, P.W. & Levine, A. (1989). *Cell*, **57**, 1083–1093.

Ginsberg, D., Mechta, F., Yaniv, M. & Oren, M. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 9979–9983.

Gorman, C., Moffat, L. & Howard, B.M. (1982a). *Mol. Cell. Biol.*, **2**, 1044–1051.

Gorman, C., Willingham, M.C., Pastan, I. & Howard, B.M. (1982b). *Proc. Natl. Acad. Sci. USA*, **79**, 6777–6781.

Gurney, E.G., Harrison, R.O. & Fenno, J. (1980). *J. Virol.*, **34**, 752–763.

Isaacs, W.B., Carter, B.S. & Ewing, C.M. (1991). *Cancer Res.*, **51**, 4716–4720.

Jenkins, J.J., Rudge, K., Chumakov, P. & Currie, G.A. (1985). *Nature*, **317**, 816–818.

Jenkins, J.J. & Sturzbecher, H.-W. (1988). In *The Oncogen Handbook*. Elsevier: Amsterdam, pp. 403–423.

Kern, S.E., Kinzler, K.W., Bruskin, A., Jarosz, D., Friedman, P., Prives, C. & Vogelstein, B. (1991). *Science*, **252** 1708–1711.

Kern, S.E., Pietenpol, J.A., Thiagalingam, S., Seymour, A. Kinzler, K.W. & Vogelstein, B. (1992). *Science*, **256** 827–830.

Lane, D.P. & Crawford, L.V. (1979). *Nature*, **278**, 261–263

Linzer, D.I.H. & Levine, A.J. (1979). *Cell*, **17**, 43–52.

Martinez, J., Georgoff, I., Martinez, J. & Levine, A. (1991) *Genes Dev.*, **5**, 151–159.

Masuda, H., Miller, C., Koeffler, H.P., Battifora, H. & Cline M.J. (1987). *Proc. Natl. Acad. Sci. USA*, **84**, 7716–7719

Mercer, W.E., Shields, M., Amin, M., Suave, G.T., Appella E., Ullrich, S. & Romano, J. (1990). *J. Cell Biochem.*, **14** 285–290.

Mercer, W.E., Shields, M.T., Lin, D., Appella, E. & Ullrich S.J. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 1958–1962.

Michalovitz, D., Halevy, O. & Oren, M. (1990). *Cell*, **62**, 671–680.

Nigro, J.M., Baker, S.J., Preisinger, A.C., Jessup, J.M., Hostetter, R., Cleary, K., Bigner, S.J., Davidon, N., Baylin, S., Devilee, P., Glover, T., Collins, F.S., Weston, A., Modali, R., Harris, C.C. & Vogelstein, B. (1989). *Nature*, **342**, 705–708.

Ohashi, Y., Sawada, Y., Moriuchi, T. & Fujinaga, K. (1992). *Biochim. Biophys. Acta*, **1130**, 175–181.

O'Rourke, R.W., Miller, C.W., Kato, G.J., Simon, K.J., Chen, D.-L., Dang, C.V. & Koeffler, H.P. (1990). *Oncogene*, **5**, 1829–1832.

Pridmore, R.D. (1987). *Gene*, **56**, 309–312.

Raycroft, L., Wu, H. & Lozano, G. (1990). *Science*, **249**, 1049–1051.

Raycroft, L., Schmidt, J.R., Yoas, K., Hao, M. & Lozano, G. (1991). *Mol. Cell. Biol.*, **11**, 6067–6074.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

Santhanam, U., Ray, A. & Sehgel, P.B. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 7605–7609.

Sarnow, P., Ho, Y.S., Williams, J. & Levine, A.J. (1982). *Cell*, **28**, 387–394.

Scheffner, S.M., Werness, B.A., Huibregtse, J.M., Levine, A.J. & Howley, P.M. (1990). *Cell*, **63**, 1129–1136.

Shiio, Y., Yamamoto, T. & Yamaguchi, N. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 5206–5210.

Sidransky, D., Von Eschenbach, A., Tsai, Y.C., Jones, P., Summerhayes, I., Marshall, F., Paul, M., Green, P., Hamilton, S.R., Frost, P. & Vogelstein, B. (1991). *Science*, **252**, 706–709.

Sleigh, M.J. (1986). *Anal. Biochem.*, **156**, 251–256.

Sturzbecher, H.-W., Brain, R., Maimets, Addison, C., Rudge, K. & Jenkins, J.J. (1988). *Oncogene*, **3**, 405–413.

ubler, M.A., Martin, D.W. & Deb, S. (1992). *J. Virol.*, **66**, 4757–4762.
kahashi, T., Takahashi, T., Suzuki, H., Hida, T., Sekido, Y., Ariyoshi, Y. & Ueda, R. (1991). *Oncogene*, **6**, 1775–1778.
ravali, S., Ku, D.-H., Rizzo, M.G., Ottavio, L., Baserga, R. & Calabretta, B. (1989). *J. Biol. Chem.*, **264**, 7466–7472.
nger, T., Nau, M.M., Segal, S. & Minna, J.D. (1992). *EMBO J.*, **11**, 1383–1390.
ang, E.H., Friedman, P.N. & Prives, C. (1989). *Cell*, **57**, 379–392.
Weintraub, H., Hauschka, S. & Tapscott, S.J. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 4570–4571.
Werness, B., Levine, A.J. & Howley, P.M. (1990). *Science*, **248**, 76–79.
Yew, P.R. & Berk, A.J. (1992). *Nature*, **357**, 82–85.
Zantema, A., Schrier, P.I., Davies-Olivier, A., Van Laar T., Vressen, R.T.M.J. & van der Eb, A.J. (1985). *Mol. Cell. Biol.*, **5**, 3084–3091.
Zhang, X.L., Bellett, A.J.D., Tha Hla, R. & Braithwaite, A.W. (1990). *Virology*, **180**, 199–206.

p53 represses SV40 transcription by preventing formation of transcription complexes

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There is now much evidence to suggest that the p53 tumour suppressor protein functions to monitor the integrity of the genome. When DNA damage is detected, p53 suppresses cell growth to allow repair or directs the cell into apoptosis. The mechanism of action of p53 is as yet unclear but recent evidence has accumulated to suggest that p53 might act by regulating gene expression. Consistent with this model, p53 can both activate and repress a number of viral and cellular promoters. p53 has also been shown to bind to the CCAAT-binding Factor and TATA-binding protein (TBP), and there is direct evidence that p53 represses *in vitro* transcription by preventing TBP from binding DNA. We now provide evidence that p53 can repress transcription from the SV40 promoter by disrupting DNA/protein complexes involving transcription factor Sp1.

Keywords: p53/SV40/gene repression

Introduction

The p53 tumour suppressor gene encodes a nuclear phosphoprotein that appears to be functionally inactivated in an extraordinarily wide range of cancers (Hollstein *et al.*, 1991). The most frequent form of this inactivation is mutation which results in altered conformation of the p53 protein (Levine *et al.*, 1991). In fact, most mutations occurring in tumours result in the protein adopting a new and common 'mutant' conformation (Gannon *et al.*, 1990). An alternative form of inactivation occurs when p53 forms stable complexes with other proteins such as that which occurs in some cervical carcinomas (Vousden, 1993). In these cases, p53 remains wild type (wt) but is inactivated by binding to the E6 protein of oncogenic human papilloma viruses (Scheffner *et al.*, 1990; Vousden, 1993). The inactivation of p53 function is so widespread that it is now regarded as a hallmark of human malignancy.

The current and most powerful model of wt p53 function is one in which p53 monitors the genome for DNA damage (Lane, 1992). If damage is detected, p53 slows cell growth (Baker *et al.*, 1990; Kuerbitz *et al.*, 1992) and activates DNA repair processes. In cancers, this monitoring process does not occur due to p53 inactivation. Thus, cells can continue to divide with damaged chromosomes. There is also evidence that in certain cell types p53 can cause apoptosis or programmed cell death in response to DNA damage caused by chemical agents and radiation (Clarke *et al.*, 1993; Lowe *et al.*, 1993).

There is now a considerable body of evidence that p53 can act as a transcription factor, which provides the basis for a potential mechanism by which p53 can inhibit cell growth (El-Deiry *et al.*, 1993) and possibly apoptosis (Shen and Shenk, 1994). In this regard, p53 has been shown to both activate and repress a large number of viral and cellular promoters in reporter molecule assays (Ginsberg *et al.*, 1991; Santhanum *et al.*, 1991; Chin *et al.*, 1992; Kley *et al.*, 1992; Shiio *et al.*, 1992; Subler *et al.*, 1992; Agoff *et al.*, 1993; Jackson *et al.*, 1993, 1994; Ueba *et al.*, 1994; Miyashita *et al.*, 1994). Importantly, expression of the negative regulator of cell cycle progression, WAF1/CIP1 (El-Deiry *et al.*, 1993; Harper *et al.*, 1993), is activated by p53.

Although the molecular basis of transcriptional regulation by p53 is not yet fully understood, the pattern of results from many studies has indicated that activation of promoters requires p53 binding to specific DNA sequences. In contrast, repression of transcription appears to occur in the absence of p53 binding to DNA (see above references). Consistent with this idea, *in vitro* studies have demonstrated that wt p53 binds directly to the CCAAT-binding Factor (CBF; Agoff *et al.*, 1993) and TATA-binding protein (TBP; Seto *et al.*, 1992; Truant *et al.*, 1993) suggesting that p53 might repress transcription by interacting with these and possibly other transcription factors to prevent their binding to promoters. Indeed, this has been demonstrated directly in experiments where purified p53 was shown to prevent TBP from binding to its site in the c-*myc* promoter (Ragimov *et al.*, 1993).

In this paper, using the SV40 early enhancer/promoter as a model, we provide evidence that p53 prevents the formation of DNA/protein complexes involving transcription factors Sp1, AP-2 and TBP. Specifically, our data demonstrate that p53 can repress transcription by directly preventing the Sp1 transcription factor from binding to its target site within the SV40 promoter.

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Results

Location of a p53-responsive element within the SV40 promoter

To identify a region within the SV40 promoter that is essential for p53 repression, we investigated the effect of p53 on the intact SV40 enhancer/promoter and on two promoter deletion constructs linked to the CAT reporter gene (Figure 1). Experiments were carried out by transfecting a number of different cell types with control and p53 expression plasmids along with appropriate reporter constructs. Qualitatively similar results were obtained in all cases. Thus the data obtained for HeLa cells only is shown in the following experiments.

As previously reported (Jackson *et al.*, 1993), activity from the intact SV40 enhancer/promoter was 20-fold lower in cells co-transfected with CMV Nc9 (expressing wt mouse p53) than in cells transfected with a control plasmid (CMV neo) or with a plasmid expressing a p53 mutant (CMV dl 163) (Table 1) which lacks part of the N-terminal transactivation domain. Expression of this mutant p53 construct has been examined many times in different cell types including HeLa, COS and rat embryo fibroblasts. In all cases the detectable p53 protein levels are at least comparable to those of a wt p53 expression construct. Examples of such data as determined by immunoprecipitation and immunoblotting have already been reported (Sturzbecher *et al.*, 1988b; Jackson *et al.*, 1993). Thus, the failure to repress transcription by dl 163 is not due to insufficient protein, but due to a specific defect in the protein. Furthermore, wt but not mutant p53, also repressed transcription from each of the SV40 promoter deletion constructs (Table 1). We conclude from these experiments that wt mouse p53 specifically represses the SV40 promoter and that since the minimum region of

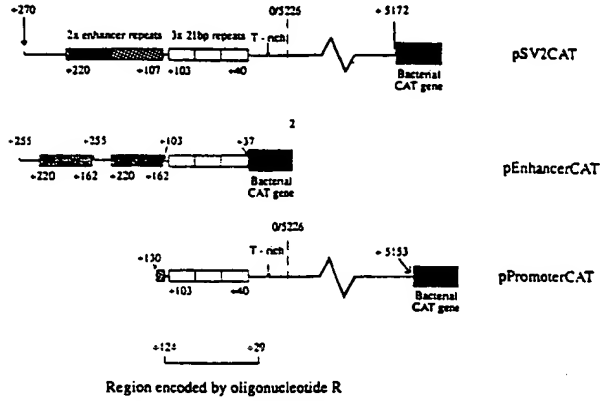


Figure 1 Map of SV40 promoter/reporter constructs used in chloramphenicol acetyl transferase (CAT) assays. Numbers refer to the nucleotide position on the SV40 DNA early strand as previously defined (Tooze, 1980). CAT activity data obtained using these constructs is illustrated in Table 1

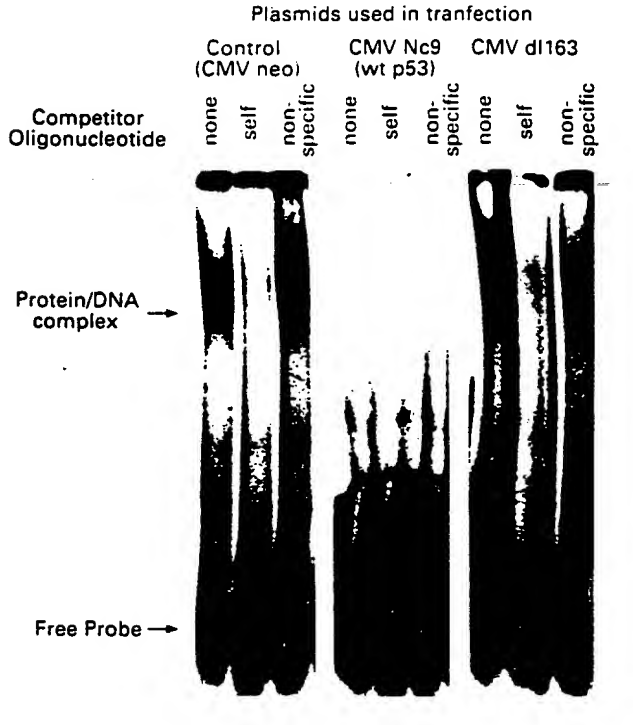


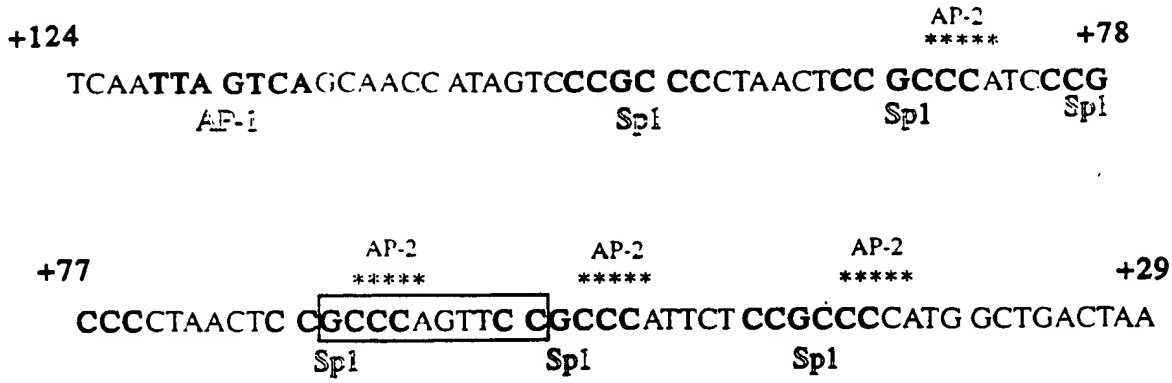
Figure 2 Wild type but not mutant mouse p53 inhibits DNA/protein complex formation. Nuclear extracts from HeLa cells transfected with CMV neo, CMV Nc9 or CMV dl 163 were incubated with ^{32}P-labelled oligo R (see below). This sequence contains a region sensitive to repression by wt mouse p53 (see Table 1). Binding reactions contained either no competitor, a 20-fold excess of unlabelled oligo R, or an unlabelled non-specific oligomer. Protein.DNA complexes were resolved on a 5% polyacrylamide gel and the positions of the specific oligo R/protein complexes and unbound probe are indicated with arrows

Table 1 Down-regulation of transcriptional activity from elements of the SV40 promoter by wt but not mutant mouse p53

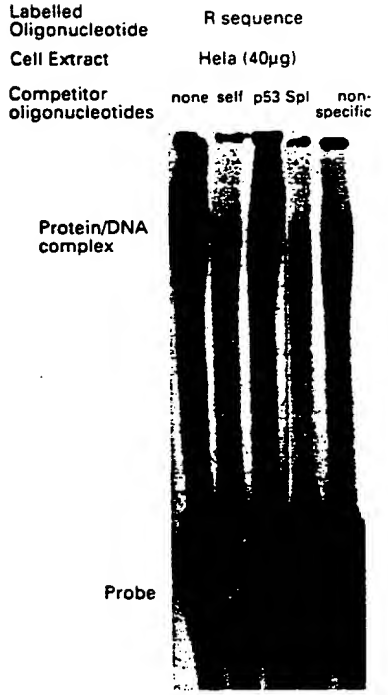
Plasmids used in co-transfection	*No of experiments*	*Relative activity* (±s.d.)	*-fold effect of p53*
pSV2 CAT+CMV neo	7	100	
pSV2 CAT+CMV Nc9	7	4.7±5.1	20-fold repression
pSV2 CAT+CMV dl 163	1	140	no repression
pEnhancer CAT+CMV neo	8	100	
pEnhancer CAT+CMV Nc9	8	14.2±11.4	sevenfold repression
pEnhancer CAT+CMV dl 163	2	194	no repression
pPromoter CAT+CMV neo	6	100	
pPromoter CAT+CMV Nc9	6	6.4±3.3	17-fold repression
pPromoter CAT+CMV dl 163	2	109	no repression

Results of CAT analysis from cells transfected with SV40 promoter/reporter plasmids (see Figure 1) and either a control plasmid (CMV neo), CMV Nc9 (expressing wild type mouse p53) or CMV dl 163 (expressing a p53 deletion mutant lacking residues 14–66). Data represented are the mean with standard deviations of several experiments except for SV2CAT+CMV dl 163 which is the result of a single experiment since we have already reported that CMV dl 163 fails to down-regulate the SV40 enhancer/promoter (Jackson *et al.*, 1993)

a



b



c

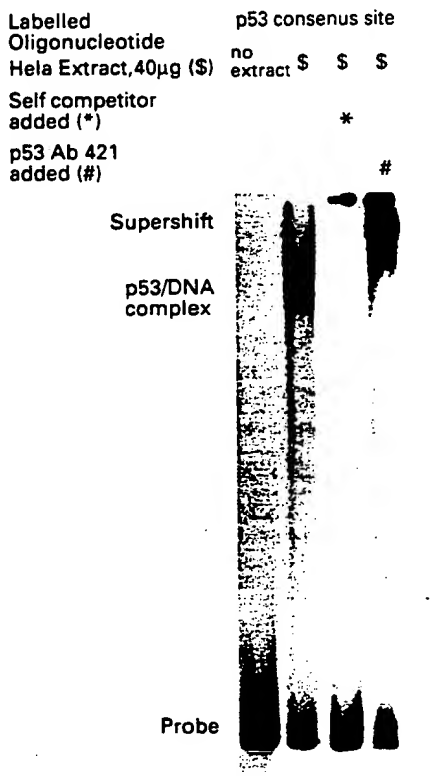


Figure 3 Multiple transcription factors bind to the R sequence of SV40 DNA but not p53. (**a**) The sequence of SV40 DNA from +29 to +124 (R sequence) with binding sites of transcription factors AP-1, AP-2 and Sp1 indicated. The boxed region indicates the putative p53 binding site reported by Bargonetti *et al.* (1991); (**b**) Sp1 but not endogenous HeLa cell p53 can bind to oligo R. Control HeLa cell extracts were incubated with ^{32}P-labelled oligo R with and without an excess of different competitor oligomers. There was a 50-fold excess of oligo R and Sp1 and a 200-fold excess of the p53 consensus oligomer (Kern *et al.*, 1991); (**c**) Endogenous HeLa p53 can bind a p53 consensus oligomer. Control HeLa cell extracts were incubated with a ^{32}P-labelled p53 oligomer in the absence or presence of a 50-fold excess of specific competitor, and in one instance in the presence of 500 ng of the p53 reactive antibody PAb 421 (Harlow *et al.*, 1981). Specific protein/DNA complexes were resolved on a 5% polyacrylamide gel and the positions of bound and free probe are indicated

overlap within the promoter deletion constructs is +37 to +130 (numbering from Tooze, 1980) (Figure 1), this region must contain a DNA sequence sufficient for p53-mediated repression.

Mouse p53 inhibits protein factors binding to the p53-response element

Since a number of transcription factors are predicted to bind to the region +37 to +130 within the SV40 promoter (Figure 3a), repression of the promoter might be due to p53 interfering with the binding of such factors to this DNA sequence. Such a mechanism has been reported to explain the repression of the c-*myc* promoter by p53 (Ragimov *et al.*, 1993). This interference could occur by direct interaction with transcription factors or by competition for DNA binding sites. To investigate these possibilities, the sequence +29 to +124 (defined as sequence R; see Figure 3a) was amplified by the polymerase chain reaction (PCR) and then used in gel mobility shift assays. Nuclear extracts were prepared from HeLa cells

separately transfected with CMV neo, CMV Nc9 expressing wt mouse p53, or CMV dl 163 encoding the mutant p53 which failed to repress the SV40 promoter in the above reporter assays (Table 1).

Results (Figure 2) showed there to be a broadly migrating R sequence/protein complex present in extracts of cells transfected with CMV neo and CMV dl 163, suggesting that the complex is composed of multiple protein factors. However, this complex was barely detectable in extracts of cells transfected with CMV Nc9 (Figure 2). These data imply that wt mouse p53 in some way prevents the formation of protein/DNA complexes involving the R sequence. This result was reproducible using several different preparations of p53-containing nuclear extracts although the degree of complex inhibition varied between different preparations, presumably due to differences in transfection efficiency. Interestingly, we did not detect binding of p53 to the R sequence in this or other experiments, despite a report that both mouse and human recombinant p53 bind a site within the R sequence (Bargonetti *et al.*, 1991). This is explored in more detail below.

Multiple trancription factors but not p53 bind to the p53-response element

The sequence of the p53-responsive region from +37 to +130 included in the R sequence (Figure 3a) contains consensus AP-1 and Sp1 transcription factor binding sites (Dynan and Tjian, 1983; Chiu *et al.*, 1987), several low affinity AP-2 binding sites (Mitchell *et al.*, 1987) and a putative p53-binding site (Bargonetti *et al.*, 1991), but is upstream of a T-rich TATA-like sequence (Pauly *et al.*, 1992). Thus, given the data in Figure 2, a likely mechanism to account for the observed repression of the SV40 enhancer/promoter is that p53 binds to one or more of the above transcription factors and prevents binding to their target sites within the promoter. Since p53 can form self oligomers (Sturzbecher *et al.*, 1992), the transfected mouse p53 might be binding the endogenous p53 and disrupting or preventing assembly of the appropriate transcriptional initiation complex on the promoter. To determine if this was a possible mechanism, we took advantage of previous observations that HeLa cells express a low level of wt p53 protein, [unpublished data, Lehman *et al.* (1991) for sequence] which is capable of binding a p53 consensus oligomer (Hoppe-Seyler and Butz, 1993; Jackson *et al.*, 1995), to ask whether p53 can bind the R sequence under our conditions.

Competition gel shift experiments were performed using oligomers corresponding to consensus binding sites for p53 and Sp1 and using control HeLa cell extracts. Results (Figure 3b) showed that a p53 consensus oligomer, surprisingly, caused no reduction in R sequence/protein complex whereas self and Sp1 oligomers caused near complete loss of complex (Figure 3b). These data indicate that Sp1 is bound to the R sequence but that p53 is not. Other competition studies indicated that AP-1 and AP-2 are also present in the R sequence complex as expected from the sequence (data not shown).

Since these competition experiments indicated that p53 was not bound to the R sequence, a p53 consensus oligomer was radiolabelled and used as a probe in a gel shift experiment to determine whether our conditions allowed DNA binding by p53. Results (Figure 3c) showed there to be clear p53 binding activity which is able to be 'super shifted' with the p53 specific monoclonal antibody PAb 421 (Harlow *et al.*, 1981). Similar results have been obtained with nuclear extracts of mouse cells (data not shown). Thus, the nuclear extracts we have used do contain p53 proteins capable of binding a consensus p53 site under our binding conditions, but which are not capable of binding the R sequence.

These data suggest that either (1) the R sequence is defective for p53 binding, (2) only recombinant p53 can bind within the R sequence, or (3) the binding of other transcription factors prevents p53 binding. The first possibility is unlikely to be correct because sequencing the amplified R fragment showed the sequence to be correct. It therefore seems likely that p53 fails to bind its putative site within the R sequence because other factors in some way interfere with its binding. This could occur by a combination of steric effects and by p53 being already bound to protein factors present in the extract. Presumably, the transfected mouse p53 also does not bind DNA (Figure 2) because it associates with protein factors in the extract, titrating out both its own DNA binding and that of other transcription factors. Significantly, the failure of p53 to bind to its site within the R sequence would be expected because p53 represses transcription from this promoter, whereas it is usual to obtain transactivation when p53 binds DNA (reviewed in Donehower and Bradley, 1993).

p53 prevents TBP, Sp1, AP-2 but not AP-1 from binding DNA

To investigate which of the above transcription factors other than endogenous p53 might be the target for wt mouse p53, consensus oligomers corresponding to the binding sites for each of the above transcription factors were used in gel mobility shift assays. Again, these experiments were carried out using extracts of HeLa cells transfected with CMV neo (conrol), CMV Nc9 and CMV dl 163.

The first set of experiments was carried out with the consensus oligomer for TBP because p53 has been reported to prevent TBP binding its DNA motif (Ragimov *et al.*, 1993). Results in Figure 4a showed a major and a minor protein/DNA complex present in extracts of cells transfected with control and mutant p53 plasmids. Both complexes were substantially reduced in extracts containing wt mouse p53. This result therefore confirms the previous report that mouse p53 can prevent formation of a TBP/DNA complex.

When a similar experiment was carried-out with a consensus oligomer for transciption factor AP-1 (Figure 4b), a single retarded DNA protein complex was evident in all three extracts. Thus, unlike the results with TBP, the data with the AP-1 consensus oligomer suggest that mouse p53 cannot prevent formation of AP-1/DNA complexes. This result has been confirmed in mixing studies using *in vitro* translated p53 and AP-1 proteins (unpublished observations).

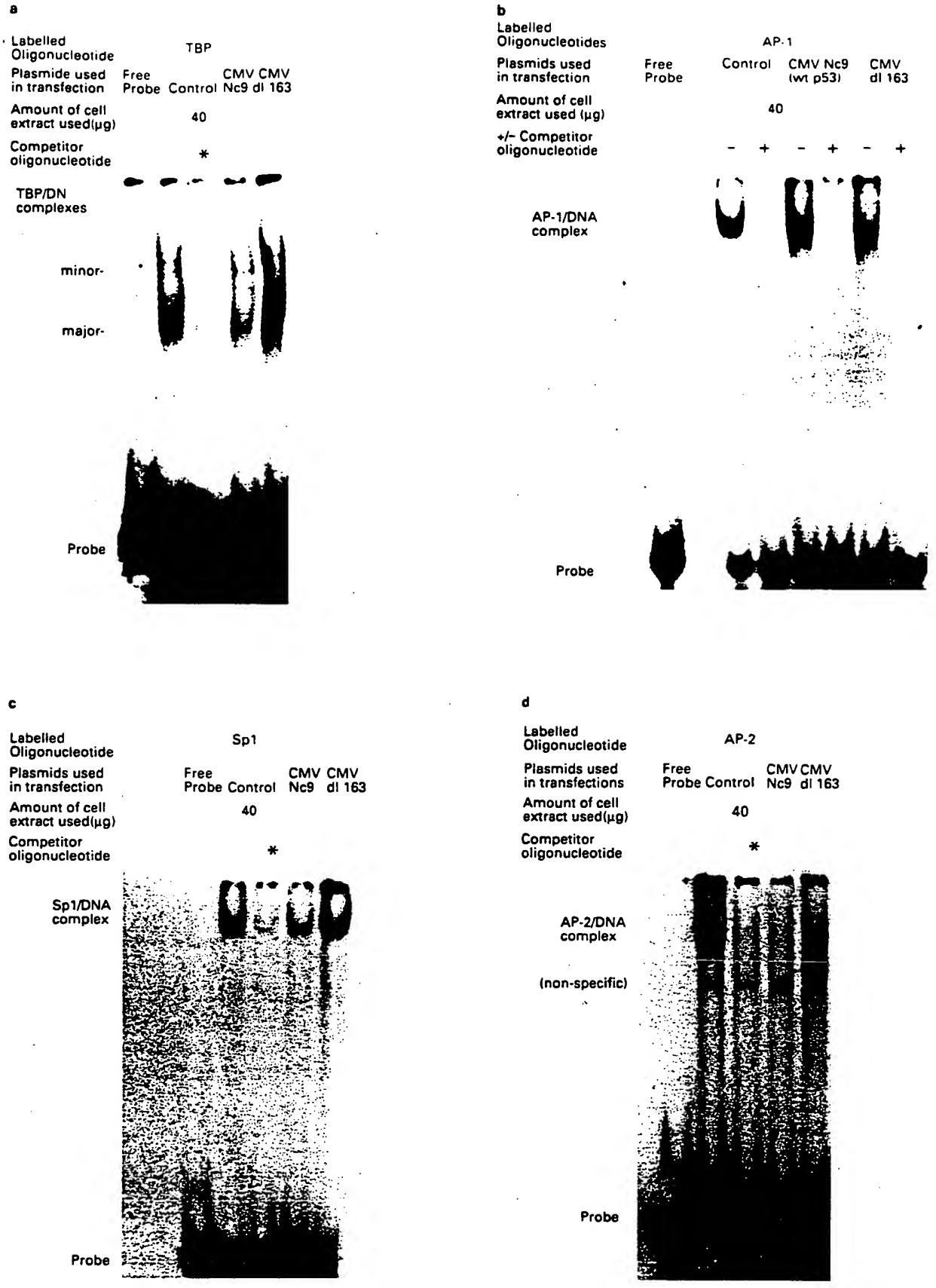
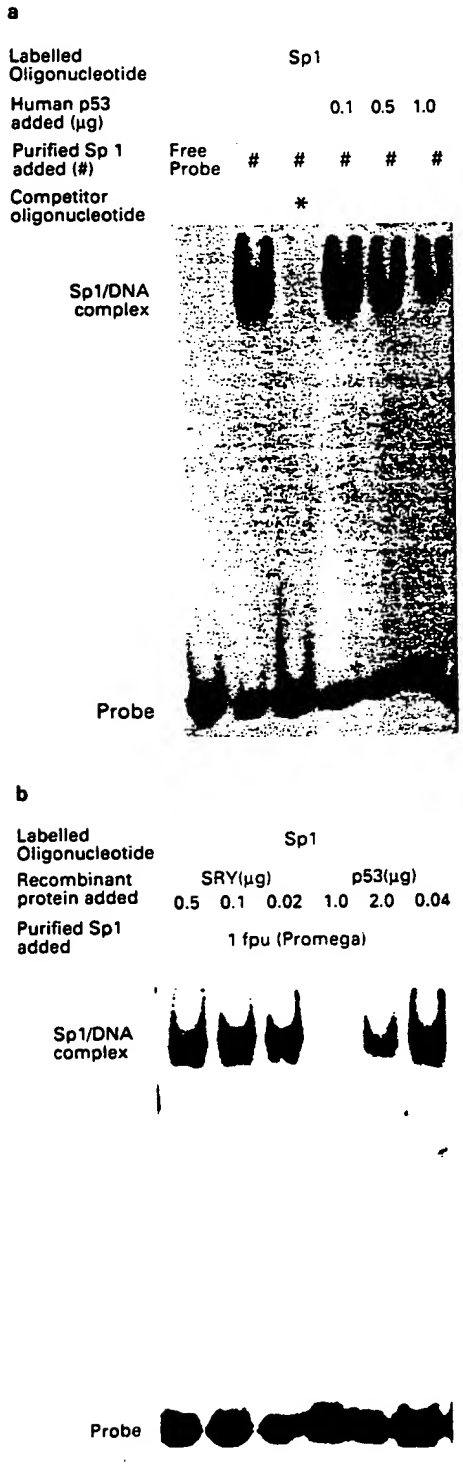


Figure 4 Wild type p53 inhibits protein/DNA complex formation involving AP-2. Sp1 and TBP. Nuclear extracts from transfected cells were mixed with ^{32}P-labelled oligomers corresponding to the binding sites for transcription factors TBP (a), AP-1, (b), Sp1 (c) and AP-2 (d). Binding reactions contained no specific competitor or a 36-fold (AP-1), 25-fold (Sp1), 36-fold (TBP) or 100-fold (AP-2) excess of the same unlabelled oligomer (tracks indicated by an asterisk). Specific protein/DNA complexes were resolved on a 5% polyacrylamide gel. Although not shown here, for all studies, irrelevant control oligonucleotide competitors have been used to validate the specificity of the binding reactions

Consensus oligomers for the binding sites for Sp1 were also used in gel mobility shift assays with transfected cell extracts. For Sp1, wt mouse p53 containing extracts showed considerably reduced binding compared to control or mutant p53 transfected extracts (Figure 4c), similar to the findings with the TBP binding site.

However, in a similar experiment with the AP-2 consensus site, whilst wt p53 completely abolished binding compared with control extracts, mutant dl 163 containing extracts also caused a substantial inhibition of binding (Figure 4d). This result suggests that p53 interacts with AP-2 in a different way from TBP and Sp1.



These data suggest that wt p53 can inhibit the binding of Sp1, AP-2 and TBP but not AP-1 to their respective DNA binding sites. In addition, mutant p53 dl 163, also appears to inhibit AP-2 binding but is unable to inhibit the binding of any of the other transcription factors.

p53 directly inteferes with Sp1 but not AP-2 binding

The results of experiments shown above suggest that p53 directly interferes with the binding of certain transcription factors to the SV40 promoter thereby causing repression of transcription. However, the data do not exclude the possibility that p53 induces expression of some intermediate protein which is responsible for interference with transcription factor binding. As the minimum response sequence (R sequence, Figure 3a) does not contain a TBP binding site and p53 is unable to inhibit AP-1 binding, only studies of Sp1 and AP-2 binding were expanded upon. To distinguish between the possibilities that p53 may interact directly with these transcription factors, or may act through another factor, gel mobility shift experiments were set up using purified recombinant human Sp1, AP-2 and p53 proteins. In this instance, human wt p53 was used instead of mouse p53 which was used in the transfection experiments. Importantly, however, CAT assays have shown that human p53 expressing constructs also repress transcription from

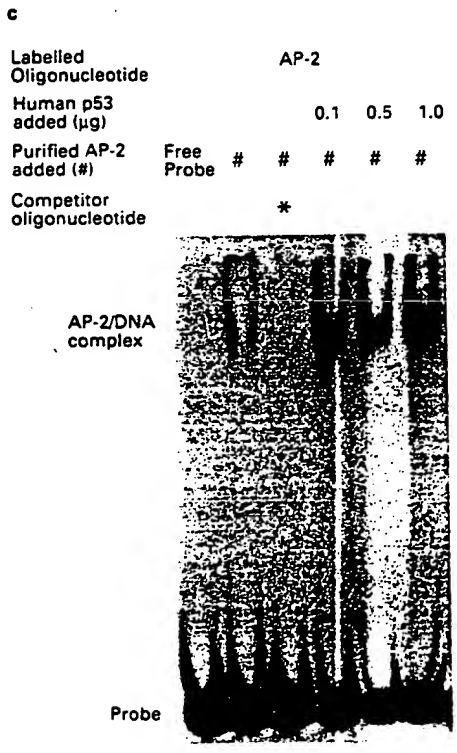


Figure 5 Recombinant p53 prevents Sp1 but not AP-2 binding DNA. Binding of purified Sp1 (1 foot printing unit (fpu), Promega) to its DNA consensus sequence was measured in the presence of increasing amounts of recombinant human p53 (**a**,**b**) or the sex-determining factor SRY (**b**). Binding of purified AP-2 (1 fpu, Promega) was also measured in the presence of increasing amounts of recombinant human p53 (**c**). Specific competitor was again present in tracks marked by an asterisk. Purified proteins were incubated in the presence of 0.05 μg of the synthetic polymer poly(dI-dC).poly(dI-dC)

the SV40 promoter although less potently (data not shown).

Consistent with our findings using transfected cell extracts, the results of gel shift experiments showed that recombinant wt human p53 interfered with the binding of purified Sp1 to its binding site in a dose dependent manner (Figure 5a and b). However, over the same molar concentration range, the HMG box binding protein SRY (sex-determining factor), which has transcriptional regulatory properties (Cohen *et al.*, 1994), did not prevent Sp1 from binding DNA (Figure 5b). These data strongly suggest that p53 and not some intermediate, is directly and specifically responsible for preventing Sp1 from binding DNA. Although quantitatively more recombinant p53 is used in these experiments than is present in the nuclear extracts used in Figure 4, there is sufficient p53 in the extracts to prevent the same amount of recombinant Sp1 binding DNA (data not shown) as was used in Figure 5. The reason for the quantitative difference is most likely because recombinant p53 is not as potent as cellular p53.

When similar experiments were carried-out with recombinant AP-2, its binding was not prevented by addition of recombinant p53 protein to the binding reaction (Figure 5c). This suggests that p53 is probably not directly responsible for interfering with AP-2 binding.

Discussion

Experiments reported in this paper have investigated a possible mechanism for transcriptional repression mediated by p53 using the SV40 enhancer/promoter as a simple and well-defined model. With the use of promoter/reporter constructs (Figure 1) we identified a region of 93 bp, from +37 to +130, which contains a DNA element sufficient for p53-mediated repression (Table 1). A PCR generated DNA fragment encompassing most of this region (R sequence, Figure 3a) was then used in gel mobility shift assays using nuclear extracts from HeLa cells transfected with CMV neo, CMV Nc9 expressing wt mouse p53 and CMV dl 163 expressing a mutant p53 which fails to repress the SV40 reporter constructs (Table 1). Control and mutant p53 containing extracts showed a broadly migrating R sequence/protein complex (suggesting the presence of multiple transcription factors) which was essentially absent from extracts containing wt mouse p53 (Figure 2).

These two sets of data, combined with the observations that the R sequence contains binding sites for several transcription factors (Figure 3a) suggested that p53 represses the SV40 promoter by interfering with transcription factors binding to promoter elements within this sequence. Competition gel shift experiments using oligomers corresponding to the consensus binding sites for all the predicted transcription factors, showed that Sp1, AP-1 and AP-2 were bound to the R sequence, but p53 was not (Figure 3b), despite the fact that the extracts did contain p53 proteins competent to bind a p53 consensus site under our conditions (Figure 3c). Thus, it seemed likely that the other transcription factors were in some way preventing the binding of p53 to its site in the R sequence by steric interference and or by direct binding of p53. These other transcription factors thus seemed likely targets for the introduced mouse p53 and again probably account for the failure of the transfected p53 to bind the R sequence.

The possibility that mouse p53 targets one of these other factors was tested using gel shift experiments with transfected cell extracts and radiolabelled oligomers corresponding to the binding sites for TBP, Sp1, AP-1 and AP-2. The results from these experiments showed that wt p53 containing extracts inhibited binding of all these transcription factors with the exception of AP-1 (Figure 4a–d).

These data therefore suggest that repression of the SV40 enhancer/promoter is due to interference by wt p53 in transcription complex formation involving the DNA sequence +37 to +130 and at least one of the transcription factors AP-2 or Sp1 (there is no TBP site within our defined region of +37 to +130). Sp1 would seem to be the more likely candidate as its DNA binding is prevented by wt p53 containing extracts (Figure 4c), but not by extracts containing the mutant p53 (Figure 4c) which fails to repress the SV40 promoter (Figure 1). In addition, p53 appears to directly interfere with Sp1 binding as indicated from studies with purified proteins (Figure 5a and b). AP-2 could also be a candidate as it too is prevented from binding to its site by wt p53 (Figure 4d). However, the fact that the mutant which does not repress the SV40 promoter, does prevent AP-2 binding, suggests that loss of AP-2 binding does not compromise promoter activity. Thus, given the above considerations, the simplest explanation of our data is that p53 represses transcription from the SV40 promoter by binding Sp1 and preventing it from binding DNA. Consistent with this interpretation, Sp1 binding has been shown to be critical for activity of the SV40 promoter (Dynan and Tjian, 1983) and also has been found to coimmunoprecipitate with p53 (Borellini and Glazer, 1993) indicating the two proteins can form a complex.

The observation that the R sequence complex is completely absent from wt p53 containing extracts (Figure 2) even though only Sp1 appears to be targeted by p53 is paradoxical. A possible explanation for this, is that Sp1 is the 'keystone' of the complex so that if this factor is lost, the complex is completely disrupted. This explanation is consistent with the competition experiments in which an Sp1 oligomer caused near complete loss of R sequence complex (Figure 3b).

Examination of the promoter sequences of the IL6 (Ray *et al.*, 1988), β-actin (Quitschke *et al.*, 1989), HIV LTR (Nabel *et al.*, 1988) and HSP 70 (Wu *et al.*, 1986) gene promoters has also identified a combination of AP-2, Sp1 and TBP binding sites. Interestingly, all of these promoters are repressed by p53. p53 can also repress a separate class of promoters which are TATA-less, for example the p53 promoter itself (Bienz-Tadmor *et al.*, 1985; Ginsberg *et al.*, 1991), and which possess GC-rich sequences, containing potential Sp1 and AP-2 binding sites. Our data therefore suggest that interference in binding of AP-2, Sp1 and/or TBP is a common mechanism for repression of basal promoter activity by p53. Such interactions may well be sufficient to explain how p53 is able to repress such a diverse collection of both cellular and viral gene promoters.

Materials and methods

Cell culture

HeLa cells (clone ATCC CCL2) were routinely maintained in Minimal Essential Medium (MEM; Gibco BRL, Grand Island, New York, USA) containing 0.22% sodium bicarbonate and 10% foetal calf serum in a humidified atmosphere with 5% CO_2 at 37°C.

Plasmids

Plasmids SV_2CAT (Gorman *et al.*, 1982), CMV neo (Braithwaite *et al.*, 1987) containing the Tn5 transposon conferring resistance to the neomycin/kanamycin family of antibiotics (Southern and Berg, 1982), pEnhancer CAT and pPromoter CAT (pCAT-enhancer and pCAT-promoter; Promega), CMV Nc9 expressing wild type mouse p53 (Eliyahu *et al.*, 1989) and CMV dl 163 expressing a mouse p53 mutant deleted between amino acids 13 and 67 (Jenkins *et al.*, 1985; Braithwaite *et al.*, 1987; Sturbecher *et al.*, 1988a) have already been described. CMV dl 163 expresses a stable protein product in transfected HeLa (Jackson *et al.*, 1993) and COS cells (Sturzbecher *et al.*, 1988b) to at least wt levels.

Transfections

For chloramphenicol acetyl transferase (CAT) assays, about 5×10^5 HeLa cells were seeded into 90 mm dishes and subsequently transfected using a modified calcium phosphate method (Chen and Okayama, 1987) with 10 μg of reporter and 10 μg of p53-expression plasmid. For gel mobility shift assays, about 3×10^6 cells were seeded into 75 cm^2 flasks and transfected using the same procedure but with 30 μg of expression plasmid and 25 μg of carrier DNA. 72 h after transfections, cells were extracted and assayed for CAT activity or used in gel shift assays. Details of the transfection procedure have been described previously (Jackson *et al.*, 1993).

Polymerase chain reaction (PCR)

Oligonucleotide R containing the putative p53-response element was amplified by PCR using pPromoterCAT as a template with a 25 bp 5'primer, 5'GGAGCTTTCAATTAGTCAGCAACCA-3', a 26 bp 3' primer, 3'-GCGGGGTACCGACTGATTGACGTCGG-5' and Taq polymerase (Pharmacia) used exactly as described by the manufacturer.

CAT assays

CAT activity was measured as previously described (Sleigh, 1986) and the details of our procedures have been reported previously (Jackson *et al.*, 1993). All assays were normalised for protein content.

Gel mobility shift experiments

HeLa cells transfected as described with CMV neo, CMV Nc9 or CMV dl 163, as well as nontransfected controls, were incubated for 72 h at 37°C and nuclear extracts prepared (Schreiber *et al.*, 1989). Once prepared, extracts were dialyzed overnight against 500 volumes of 20 mM Tris-HCl pH 7.0, 10% glycerol, 1 mM EDTA, 40 mM NaCl, 1 mM DTT (TEG). Gel mobility shift assays were performed by incubating 40 μg of nuclear extract with 10 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 12.5% glycerol, 0.1% Triton-X-100 (Borellini and Glazer, 1993) and 1 μg of the synthetic polynucleotide poly(dI-dC).poly(dI-dC) for 15 min at 20°C. Unless otherwise indicated, during this time any competitors were also included. Labelled oligonucleotide probe (10 000 c.p.m.) was added and the reaction continued for a further 15 min. Protein/DNA complexes were then resolved on 5% polyacrylamide gels which were fixed in 10% acetic acid, dried under vacuum at 80°C for 25 min and exposed to Kodak XAR-5 film at −70°C.

When binding reactions were carried-out using purified recombinant human AP-2, Sp1 (both purchased from Promega) and p53 (see below), conditions were as described above but without cell extract.

Purification of p53 protein

Human p53 was purified from *E. coli* containing pET19b-hup53. A single colony was inoculated into LBamp medium overnight and induced in 400 ml of LBamp with 1.0 mM IPTG at $O.D_{600}$ of 0.5. The cells were incubated at 37°C for 3 h and harvested by centrifugation at 8000 *g* for 10 min at 4°C. The cells were then lysed for 1 h at 4°C in 6 M guanidinium/HCl, 50 mM Tris-HCl, pH 8.0. The lysate was cleared by centrifugation at 12000 *g* for 20 min at 4°C and the supernatant filtered through gauze. The supernatant was then incubated overnight with 750 ml of Ni^{2+} Nitrilotriacetic acid (NTA)-agarose equilibrated with the guanidinium lysis buffer. The agarose beads were pelleted at 1000 *g* and washed five times with the lysis buffer. The beads were then dialysed against p53 buffer (25 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10 mM β-2-Mercaptoethanol, 0.1% Triton-X-100, 7.5% glycerol and 300 mM NaCl) containing 1 M guanidinium, subsequently 0.1 M guanidinium and then without guanidinium. Upon transfer to a column and washing with five volumes of p53 buffer (pH 6.3), 500 μl fractions were eluted with p53 buffer pH 5.0 and collected in eppendorfs containing 17 μl of 500 mM Tris-HCl, pH 8.0. Column fractions were then analysed by SDS–PAGE and the purified protein was subsequently dialysed against 500 volumes of TEG. Aliquots were stored at −70°C.

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References

Agoff SN, Hou J, Linzer DIH and Wu B. (1993). *Science*, **259**, 84–87.

Baker SJ, Markowitz S, Fearon ER, Willson JKV and Vogelstein B. (1990). *Science*, **249**, 912–915.

Bargonetti J, Friedman PN, Kern SE, Vogelstein B and Prives C. (1991). *Cell*, **65**, 1083–1091.

Bienz-Tadmor B, Zakut-Houri R, Libresco S, Givol D and Oren M. (1985). *EMBO J.*, **4**, 3209–3213.

Borellini F and Glazer RI. (1993). *J. Biol. Chem.*, **268**, 7923–7928.

Braithwaite AW, Sturzbecher H-W, Addison C, Palmer C, Rudge K and Jenkins JR. (1987). *Nature*, **329**, 458–460.

Chen C and Okayama H. (1987). *Mol. Cell. Biol.*, **7**, 2745–2752.
Chin K-V, Ueda K, Pastan I and Gottesman M. (1992). *Science*, **255**, 459–462.
Chiu R, Imagawa M, Imbra RJ, Backoven JR and Karin M. (1987). *Nature*, **329**, 648–651.
Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML and Wyllie AH. (1993). *Nature*, **362**, 849–852.
Cohen DR, Sinclair AH and McGovern JD. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 4372–4376.
Donehower LA and Bradley A. (1993). *Biochim. Biophys. Acta.*, **1155**, 181–203.
Dynan WS and Tjian R. (1983). *Cell*, **35**, 79–97.
El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B. (1993). *Cell*, **75**, 817–825.
Eliyahu D, Michalovitz D, Eliyahu S, Pinhasi-Kimhi O and Oren M. (1989). *Proc. Natl. Acad. Sci. USA*, **86**, 8763–8767.
Gannon JV, Greaves RG, Iggo R and Lane DP. (1990). *EMBO J.*, **9**, 1595–1602.
Ginsberg D, Mechta F, Yaniv M and Oren M. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 9979–9983.
Gorman C, Moffat and Howard BM. (1982). *Mol. Cell. Biol.*, **2**, 1044–1051.
Harlow E, Crawford LV, Pim DC and Williamson NM. (1981). *J. Virol.*, **39**, 861–869.
Harper JW, Adami GR, Wei N, Keyomarsi K and Elledge SJ. (1993). *Cell*, **75**, 805–816.
Hollstein M, Sidransky D, Vogelstein B and Harris CC. (1991). *Science*, **253**, 49–53.
Hoppe-Seyler F and Butz K. (1993). *J. Virol.*, **67**, 3111–3117.
Jackson P, Bos E and Braithwaite AW. (1993). *Oncogene*, **8**, 589–597.
Jackson P, Ridgway P, Rayner J, Noble J and Braithwaite A. (1994). *Biochem. Biophys. Res. Commun.*, **203**, 133–140.
Jackson P, Shield M, Buskin J, Hawkes S, Reed M, Perrem K, Hauschka SD and Braithwaite A. (1995). *Gene Expression*, **5**, in press.
Jenkins JR, Rudge K, Chumakov P and Currie GA. (1985). *Nature*, **317**, 816–818.
Kern SE, Kinzler KW, Bruskin A, Jarosz D, Friedman P, Prives C and Vogelstein B. (1991). *Science*, **252**, 1708–1711.
Kley N, Chung RY, Fay S, Loeffler JP and Seizinger BR. (1992). *Nucl. Acids Res.*, **20**, 4083–4087.
Kuerbitz SJ, Plunkett BS, Walsh WV and Kastan MB. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 7491–7495.
Lane DP. (1992). *Nature*, **358**, 15–16.
Lehman TA, Bennett WP, Metcalf RA, Welsh JA, Ecker J, Modali RV, Ullrich S, Romano JW, Appella E and Testa JR. (1991). *Cancer Res.*, **51**, 4090–4096.
Levine AJ, Momand J and Finlay CA. (1991). *Nature*, **351**, 453–456.
Lowe SW, Schmitt EM, Smith SW, Osborne BA and Jacks T. (1993). *Nature*, **362**, 847–849.
Mitchell PJ, Wang C and Tjian R. (1987). *Cell*, **50**, 847–861.
Miyashita T, Masayoshi M, Hanada M and Reed JC. (1994). *Cancer Res.*, **54**, 3131–3135.
Nabel GJ, Rice SA, Knipe DM and Baltimore D. (1988). *Science*, **234**, 1299–1302.
Pauly M, Treger M, Westhof E and Chambon P. (1992). *Nucl. Acids Res.*, **20**, 975–982.
Quitschke WW, Lin Z-Y, Deponti-Zilli L and Paterson BM. (1989). *J. Biol. Chem.*, **264**, 9539–9546.
Ragimov N, Krauskopf A, Navot N, Rotter V, Oren M and Aloni Y. (1993). *Oncogene*, **8**, 1183–1193.
Ray A, Tatter SB, May LT and Sehgal PB. (1988). *Proc. Natl. Acad. Sci. USA*, **85**, 6701–6705.
Santhanum U, Ray A and Sehgal PB. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 7605–7609.
Scheffner SM, Werness BA, Huibregste JM, Levine AJ and Howley PM. (1990). *Cell*, **63**, 1129–1136.
Schreiber E, Matthias P, Muller MM and Schaffner W. (1989). *Nucl. Acids Res.*, **17**, 6419.
Seto E, Usheva A, Zambetti GP, Momand J, Horikoshi N, Weinmann R, Levine AJ and Shenk T. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 12028–12032.
Shiio Y, Yamamoto T and Yamaguchi N. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 5206–5210.
Shen Y and Shenk T. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 8940–8944.
Sleigh MJ. (1986). *Anal. Biochem.*, **156**, 251–256.
Southern PJ and Berg PJ. (1982). *Molec. Appl. Genet.*, **1**, 327–341.
Sturzbecher H-W, Brain R, Maimets T, Addison C, Rudge K and Jenkins JR. (1988a). *Oncogene*, **3**, 405–413.
Sturzbecher H-W, Braithwaite AW, Addison C, Palmer C, Rudge K, Lynge-Hansen D and Jenkins JR. (1988b). *Cancer Cells*, **6**, 159–163.
Sturzbecher H-W, Brain R, Addison C, Rudge K, Remm M, Grimaldi M, Keenan E and Jenkins JR. (1992). *Oncogene*, **7**, 1513–1523.
Subler MA, Martin DW and Deb S. (1992). *J. Virol.*, **66**, 4757–4762.
Tooze J. (1980). *DNA Tumor Viruses*, **Pt.2**, Tooze J. (ed.), Cold Spring Harbor Laboratory, New York.
Truant R, Xiao H, Ingles CJ and Greenblatt J. (1993). *J. Biol. Chem.*, **268**, 2284–2287.
Ueda T, Nosaka T, Takahashi JA, Shibata F, Florkiewicz RZ, Vogelstein B, Oda Y, Kikuchi H and Hatanaka M. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 9009–9013.
Vousden KH. (1993). *FASEB J.*, **7**, 872–879.
Wu BJ, Kingston RE and Morimoto RI. (1986). *Proc. Natl. Acad. Sci. USA*, **83**, 629–633.

Adenovirus-Mediated p53 Gene Transfer in Patients With Advanced Recurrent Head and Neck Squamous Cell Carcinoma

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Purpose: **Standard therapies of head and neck squamous cell carcinoma (HNSCC) often cause profound morbidity and have not significantly improved survival over the last 30 years. Preclinical studies showed that adenoviral vector delivery of the wild-type p53 gene reduced tumor growth in mouse xenograft models. Our purpose was to ascertain the safety and therapeutic potential of adenoviral (Ad)-p53 in advanced HNSCC.**

Patients and Methods: **Patients with incurable recurrent local or regionally metastatic HNSCC received multiple intratumoral injections of Ad-p53, either with or without tumor resection. Patients were monitored for adverse events and antiadenoviral antibodies, tumors were monitored for response and p53 expression, and body fluids were analyzed for Ad-p53.**

Results: **Tumors of 33 patients were injected with doses of up to 1×10^{11} plaque-forming units (pfu). No dose-limiting toxicity or serious adverse events were noted. p53 expression was detected in tumor biopsies despite antibody responses after Ad-p53 injections. Clinical efficacy could be evaluated in 17 patients with nonresectable tumors: two patients showed objective tumor regressions of greater than 50%, six patients showed stable disease for up to 3.5 months, and nine patients showed progressive disease. One resectable patient was considered a complete pathologic response. Ad-p53 was detected in blood and urine in a dose-dependent fashion, and in sputum.**

Conclusion: **Patients were safely injected intratumorally with Ad-p53. Objective antitumor activity was detected in several patients. The infectious Ad-p53 in body fluids was asymptomatic, and suggests that systemic or regional treatment may be tolerable. These results suggest the further investigation of Ad-p53 as a therapeutic agent for patients with HNSCC.**

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SQUAMOUS CELL CARCINOMAS of the head and neck (HNSCC) comprise approximately 4% of cancers in the United States and cause approximately 2% of all US cancer deaths. The principal cause of death among these patients is local-regional recurrence.[1,2] Standard therapy is frequently associated with profound speech, swallowing, and cosmetic morbidities. Although treatment advances have been made in the last 30 years, little or no survival improvement has been obtained.[3,4] New strategies are clearly needed.

The p53 tumor-suppressor gene is the most frequently mutated gene identified in human cancers, and is mutated in a majority of HNSCs.[5] p53 is a multifunctional protein that, among other activities, acts as a transcriptional activator and repressor, is induced by DNA damage, and interacts with proteins involved in DNA replication and repair.[6,7] p53 appears to have a vital role in the sensing and repair of DNA damage, inhibiting the cell cycle to allow DNA repair, or inducing apoptosis to eliminate severely damaged cells.[7]

An adenoviral vector system was chosen for a gene therapy approach because of the ability to infect many cell types, both quiescent and dividing, lack of integration into the host genome, high-level transgene expression, ease of high titer and large scale manufacture, and the established safety of adenovirus vaccines.[8,9] The vector used is com-

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posed of the wild-type p53 gene inserted into a first-generation adenoviral backbone (Ad-p53).

Preclinical studies with Ad-p53 have shown that p53 transduction can induce apoptosis and decrease cell proliferation in a number of cancer cell lines without adversely affecting normal cells.[10-13] In general, p53 gene therapy is more effective with p53 mutant cancer cell lines, but it is also active against wild-type p53 cancer cell lines.[14-17] Ad-p53 also reduces tumor growth in xenograft models of HNSCC and other cancers.[18-20] In addition, Ad-p53 potentiates cytotoxic chemotherapy and radiation therapy in model systems.[21-23]

This study was conducted to determine the safety of Ad-p53 in patients with advanced, recurrent HNSCC; to document possible antitumor activity; and to evaluate transgene expression, Ad-p53 dissemination, and shedding. The potential suitability of direct intratumoral injections of Ad-p53 was investigated in patients with advanced disease and, in one arm of the study, in a surgical adjuvant setting.

PATIENTS AND METHODS

Study Subjects

Thirty-four patients with advanced recurrent or refractory squamous cell carcinoma of the upper aerodigestive tract (Eastern Cooperative Oncology Group performance status ≤ 2) were entered onto either the resectable or nonresectable arm of the study (Table 1). Patients were entered onto the resectable arm if the tumor could be resected for debulking, but resection alone posed no chance for a cure. All patients were presented in the M.D. Anderson Multidisciplinary Head and Neck Oncology Planning Conference before they consented to this protocol. One patient withdrew before treatment and was excluded from the analysis. Nine women and 24 men, of whom 15 had resectable disease and 18 had nonresectable disease, participated in the study. They had a mean age of 54 years (range, 32 to 76 years), and 29 patients were white, two patients were black, and two patients were Hispanic. Twenty-seven patients had had prior surgery, 32 had received prior radiation, and 21 had received salvage chemotherapy (Table 1). Patients had large tumor burdens, and the vast majority had more than one lesion. Fourteen tumors from patients in the resectable arm and 12 tumors from patients in the nonresectable arm were bidimensionally measured by computed tomographic (CT) scan before the date of the first dose. The mean area of tumors in the resectable arm was 13.76 ± 13.01 cm^2 (range, 1.00 to 38.25 cm^2; n = 12) and the mean in the nonresectable arm was 17.93 ± 16.15 cm^2 (range, 4.84 to 54.60 cm^2; n = 14). The two-sample *t* test showed no statistically significant difference between these two groups with regard to tumor size ($P = .4811$). The photograph (Fig 1) shows a typical advanced recurrent-disease patient entered onto this trial, and shows the absence of curative therapeutics available to these protocol patients.

Tumor p53 gene status, either mutant or wild-type, was not an entry requirement but was determined for each patient. All women of childbearing age had negative pregnancy tests, and all patients were required to practice contraception while on the study. This protocol was reviewed and approved by the Institutional Surveillance Committee of the University of Texas M.D. Anderson Cancer Center, the National Institutes of Health Recombinant DNA Advisory Committee, and the Food and Drug Administration. Informed consent was obtained from all patients before entry onto the study.

Ad-p53

Ad-p53, designated as INGN 201, is a replication-defective adenovirus serotype 5 (Ad5) vector with a p53 cDNA expression cassette that replaces the E1 region of the virus.[24] Ad-p53 is a biosafety level 2 (BL-2) agent and was handled with the appropriate or even greater level of biologic containment. Ad-p53 was produced under Good-Manufacturing-Practices conditions at Magenta, Inc (now MA Biosciences, Rockville, MD) and stored at − 80°C at concentrations of 2 to 3.5 × 10^{10} plaque-forming units (pfu) per mL in phosphate-buffered saline supplemented with 10% glycerol. Administered Ad-p53 was free of replication-competent adenovirus at one part in 10^9 (data not shown). Ad-p53 was thawed and diluted in phosphate-buffered saline at 4°C within 2 hours of use.

Patient Treatment and Examination

Patients were sequentially enrolled at each dose level, and each patient received an assigned dose throughout the study. The dose of Ad-p53 was escalated in log increments from 10^6 to 10^9 pfu, and in half-log increments from 10^9 to 10^{11} pfu. Three to six patients were assigned to each dose level. All patients in the study received Ad-p53 therapy.

Each patient received at least one course of Ad-p53 injections. Each course consisted of Ad-p53 administration three times per week (every other day) for 2 weeks for a total of six administrations. Resectable-disease patients received only one full course of injections followed by two additional administrations; one during surgery after gross tumor removal in the site of microscopic residual disease and one 72 hours after surgery through retrograde catheter instillation. Resectable-disease patients were then observed throughout their follow-up, with no additional cycles of Ad-p53 administration. Nonresectable-disease patients underwent a 2-week rest period before the next course of Ad-p53 injections. Nonresectable-disease patients repeated the Ad-p53 courses monthly until disease progression or consent was withdrawn, for up to seven courses of treatment. Only a single site of disease was selected as the indicator lesion, even for patients with multiple sites of disease. Because the patients clearly understood the phase I nature of this investigation and the primary end points of toxicity and tolerance, efforts to inject recurrences were directed at the most accessible, assessable, and measurable masses.

Ad-p53 was injected directly into tumors, either visually or as directed by manual palpation, in a total volume of 1.5 to 10 mL, which depended on tumor volume. Patients remained under close observation for at least 2 hours after each administration. Ad-p53 injections were performed in a hospital room under respiratory and body-secretion isolation during each 2-week cycle, and the medical staff used reverse isolation procedures, which included the use of a HEPA-filtered mask. Patients remained under respiratory isolation for the 2-week investigation period until 72 hours after the last injection. Tumors were injected with a total volume based on the number of injection sites, which were spaced in 1-cm increments over the clinically assessed indicator lesion. Between 0.5 and 1.0 mL of Ad-p53 was delivered to each injection site, based on the third dimension (depth) of the mass as determined by clinical assessment.

Vital signs, hematology, chest radiography, blood chemistry, and performance status were monitored at the beginning of each treatment cycle. All adverse events reported during the study were evaluated and graded on a scale of 1 to 4. The National Cancer Institute Common Toxicity Criteria (NCI-CTC)[25] were used to determine the grade for all toxicity listed on the scale. For adverse events not listed on the NCI-CTC, the following system was used: grade 1, mild; grade 2, moderate; grade 3, severe; and grade 4, life-threatening. Ad-p53

Table 1. Patient Profile

Patient No.*	Age (years)	Sex	p53 Mutations†	p53 Immuno-staining	Prior Failed Therapies	Primary Cancer	Site of Injection	Treatment Courses‡	PFU Per Injection	Study Arm	Clinical Activity
1	32	F	arg175 to his	+	Surgery, xrt, chemo	Floor of mouth	Neck	2	10^8	R	NA
2	59	M	arg267 to pro	+	Surgery, xrt	Larynx	Neck	2	10^6	R	NA
3	57	M	ser127 to tyr	+	Surgery, xrt, chemo	Pyriform sinus	Left neck mass	1	10^6	NR	PD
4	43	F	WT	+	Surgery, xrt	Base of tongue	Right neck mass	2§	10^6	NR	PD
5	73	M	WT	−	Surgery, xrt	Unknown	Left neck mass	1	10^7	R	CR
6	46	M	leu257 to gln	+	Surgery, xrt, chemo	Cervical, esophagus	Suprastomal lesion	1	10^6	NR	SD
7	64	M	WT	−	Xrt, chemo	Tonsil	Left neck mass	5§	10^7	NR	SD
8	47	M	arg248 to pro	+	Surgery, xrt, chemo	Base of tongue	Base of tongue	1	10^7	R	NA
9	58	M	WT	+	Surgery, xrt, chemo	Larynx	Peristomal area	1	10^7	NR	PD
10	58	M	arg282 to trp	ND	Surgery, xrt	Larynx	Left hypopharynx mass	1	10^8	R	NA
11	49	M	tyr236 to cys	+	Xrt, chemo	Base of tongue	Base of tongue	1	10^9	R	NA
12	57	F	arg175 to his	+	Surgery, xrt, chemo	Floor of mouth	Left floor of mouth, mandible	3	10^8	NR	SD
13	66	M	WT	+	Surgery, xrt	Base of tongue	Right tongue, right posterior tongue	7	10^9	NR	PD
14	49	F	gln167 to stop	−	Surgery, xrt	Floor of mouth	Floor of mouth	2§	10^9	NR	PD
15	64	M	WT‖	−	Surgery, xrt, chemo	Mandible alveolar ridge	Left facial mass	1	10^9	NR	NE
16	76	F	arg248 to trp	+	Xrt	Larynx	Left supraclavicular mass	1	10^9	R	NA
17	56	M	WT	+	Surgery, xrt, chemo	Larynx	Base of tongue, left BOT, tonsil	1	10^9	NR	PD
18	57	M	WT	−	Exp	Left lateral pharyngeal wall	Left facial mass	4	10^9	NR	SD
19	54	M	WT	−	Surgery, xrt, chemo	Unknown	Right submental mass	1	3×10^9	R	NA
20	56	M	WT	−	Surgery, xrt, chemo	Tongue	Left neck mass	3	3×10^9	NR	SD
21	67	M	NE	ND	Xrt, chemo	Base of tongue	Right infraaurical area	5	3×10^9	NR	PD
22	57	M	lys132 to asn	+	Surgery, xrt, chemo	Larynx	Anterior neck, suprastomal	3	10^{10}	NR	PD
23	38	M	WT	+	Surgery, xrt	Left mandible	Left infraauricular region	6	10^{10}	NR	≥ 50% tumor regression
24	50	M	WT	−	Surgery, xrt, chemo	Left retromolar trigone	Left cheek	1	10^{10}	NR	PD
25	57	M	tyr126 to cys	+	Surgery, xrt, chemo	Pharynx	Nasopharynx	1	3×10^{10}	R	NA
26	49	F	NE¶	−	Surgery, xrt, chemo	Left oral tongue	Left lateral tongue	1	3×10^{10}	R	NA
27	67	M	cys275 to trp	+	Xrt	Left tonsil	Left tonsil	1	3×10^{10}	R	NA
28	36	M	2 bp deletion at codon 209#	ND	Surgery, xrt	Base of tongue	Left base of tongue	1	10^{11}	R	NA
29	34	F	NE	ND	Surgery, xrt, chemo	Submental area	Submental area, base of tongue	1	10^{11}	R	NA
30	32	M	WT	+	Surgery, xrt, chemo	Left superior anterior neck dermal metastasis	Left anterior superior neck dermal mass	3	10^{11}	NR	≥ 50% tumor regression
31	56	M	ala 307 to ser	−	Surgery, xrt, chemo	Right hypopharynx	Right hypopharynx mass	1	10^{11}	R	NA
32	72	F	ile 232 to ser, gln 331 to stop	+	Surgery, xrt	Left buccal mass	Left buccal mass	1	10^{11}	R	NA
34	47	F	WT	+	Surgery, xrt, chemo	Nasopharynx	Right preauricular mass	4	10^{11}	NR	SD

Abbreviations: +, positive; −, negative; WT, wild-type; NE, could not be evaluated; ND, not determined; xrt, radiation therapy; NA, not applicable; NR, nonresectable; R, resectable; PD, progressive disease; SD, stable disease; chemo, chemotherapy; Exp, experimental plant extract treatment in Europe; BOT, base of tongue; CR, complete histologic response (see text).

*Patient 33 withdrew consent before the start of treatment.

†With the one noted exception, samples listed as NA could not be assessed because of insufficient tumor cells (< 10%) in the biopsy.

‡Each course had 6 injections. Several patients had a partial final course of 1-3 injections.

§The full set of injections was not completed in the last course.

‖Exon 6 was not sequenced.

¶There was only sufficient tumor to sequence exon 6; it was wild-type.

#The resulting frameshift led to a stop codon at codon 214.

Fig 1. An aggressive recurrent squamous carcinoma of the tongue with direct extension into the neck (patient 1, resectable-disease group), which is representative of the advanced burdens of local-regional disease incurred by patients who entered onto the trial.

administrations were terminated for disease progression or inability to tolerate treatment. To avoid the enrollment of more patients than necessary onto the trial if excessive toxicity was found, a Bayesian early-stopping rule was included in the study protocol but was not implemented.

Clinical activity was evaluated by CT scan. Only nonresected patients were evaluated, by using standard criteria applied to the indicator lesion.

Sequencing of p53

Patient tumor p53 status was determined by sequencing exons 5 through 10 of the p53 gene obtained from tumor cell DNA. DNA was isolated from biopsies with a Qiagen Blood and Tissue Kit (Qiagen, Santa Clarita, CA) and polymerase chain reaction (PCR) sequencing was performed with either the AmpliCycle sequencing kit (Perkin Elmer, Norwalk, CT) or the ThermoSequenase terminator cycle sequencing kit (Amersham, Arlington Heights, IL).[26] This method allowed detection of p53 mutations if the biopsy contained 20% or greater of tumor cells.

Ad-p53 Dissemination Assays

Samples. The presence of Ad-p53 in urine, blood, and upper aerodigestive tract secretions (UATS) was assayed by cytopathic effect (CPE) and Ad-p53–specific PCR. Urine samples consisted of a first morning void that began with a pretreatment sample on the first day of treatment. Samples were collected daily during hospitalization and less regularly after discharge. Plasma was collected from Cell Preparation Tubes (Becton-Dickinson, San Jose, CA) within 4 hours of collection. Serum was obtained by standard methods. UATS consisted of expectorated sputum or saliva or a saline rinse in patients with xerostomia.

CPE assay. CPE assays were performed on 293 and A549 cells; 293 cells[27] are permissive for Ad-p53 growth and A549 cells are nonpermissive for Ad-p53 growth but permissive for growth of replication-competent adenovirus. No assays were positive for A549 CPE, which showed the lack of replication-competent adenovirus in patient samples. Plates were examined for CPE for up to 9 days. Standard curves with wild-type Ad5 (on 293 cells) and Ad-p53 (on A549 cells) were run with each assay. The CPE assay was found to be semiquantitative, with a probable error of plus or minus one order of magnitude and a sensitivity of 10 pfu per 0.5-mL sample or less. Selected 293 cell–positive CPE supernatants were assayed by an adenovirus hexon enzyme-linked immunosorbent assay (ELISA) and Ad-p53–specific PCR. Within the assay sensitivity limits, all supernatants were positive, which confirmed the identity of Ad-p53 in the patient samples.

Urine sample preparation for CPE assays included one freeze-thaw cycle, sterile filtration, and a twofold dilution in DMEM HG (Life Technologies, Gaithersburg, MD) before overlaying onto cells. After 30 minutes, two volumes of medium were added, and the plates were incubated and read as above. Patient plasma was added to 293 and A549 cells undiluted and unfiltered and scored as above. UATS samples were frozen and thawed, and homogenized vigorously by repeated pipetting and vortexing. Samples were diluted 25% to 50% with phosphate-buffered saline, clarified and sterilized with a prefilter/membrane combination (0.2-μm Serum Acrodisk filter, Gelman Sciences, Ann Arbor, MI), added to cells, and scored as above.

The hexon ELISA (Adenoclone EIA; Meridian Diagnostics, Cincinnati, OH) included controls provided by the manufacturer as well as supernatant from 293 cells infected with Ad-p53.

PCR assay. PCR was used to assay plasma, serum, urine, and UATS directly. Extreme precautions were taken to prevent PCR contamination and no evidence of such contamination was encountered. Sensitivity of the assay was 10^3 pfu per 0.5-mL sample. Oligonucleotide primers were obtained from Oligos, Etc (Wilsonville, OR).

Urine samples for PCR were frozen and thawed once, and DNA was isolated with the QIAamp HCV Kit (Qiagen). The QIAamp Tissue Kit was used to isolate DNA from 293 CPE supernatants. Primers consisted of 5′-TAGAGCCAAACTCAGCGCGG-3′ and 5′-ATCCGTGGGCGTGAGCGCT-3′. The PCR product was selected to cross a p53 open-reading–frame/adenoviral DNA junction specific for Ad-p53.

Two different blood fractions were assayed for Ad-p53. In the original protocol, serum was tested (patients 1 to 25). However, blood fractionation studies performed during the course of this trial showed enhanced sensitivity with plasma (data not shown). For patients 25 to 28, both plasma and serum were assayed. After showing equivalent results with the two assays, the remaining samples (patients 29 to 34) were assayed by using only plasma.

For PCR on serum samples, DNA was isolated with either a QIAamp Blood Kit and PCR-amplified as described above for urine, or a modification of the protocol of Cunningham et al[28] and amplified with primers 5′-CACTGCCCAACAACACCA-3′ and 5′-GCCACGCCCACACATTT-3. The PCR product was selected to cross a p53 open-reading–frame/adenoviral DNA junction specific for Ad-p53.

Plasma samples for PCR were frozen and thawed and homogenized vigorously by repeated pipetting and vortexing. DNA was extracted (QIAamp Blood Kit) and PCR-amplified as described above for urine PCR.

UATS samples for PCR were frozen and thawed, homogenized vigorously by repeated pipetting and vortexing, and DNA was extracted with the QIAamp Tissue Kit and PCR-amplified as described above for urine PCR.

Reverse-transcriptase PCR. The presence of p53 transgene mRNA in patient biopsies was detected by reverse-transcriptase (RT)-PCR. Total RNA was isolated with TRI reagent (Molecular Research, Cincinnati, OH) from pre- and posttreatment flash-frozen biopsies. The RNA was then DNAse I-treated and reverse transcribed (Superscript RNAse H⁻RT and random hexamer primers [Life Technologies]), and the resulting DNA amplified by PCR by using primers specific to Ad-p53 mRNA. (5′-GGTGCATTGGAACGCGGATT and 5′-GGGGACAGAACGTTGTTTTC). Identity of the PCR products was confirmed

by Southern blot hybridization.[28] Positive controls for glyceraldehyde-phosphate dehydrogenase (GAPDH) mRNA and negative controls (blanks, minus RT) gave the expected results (data not shown).

Anti-Ad5 Antibody Assays

Serum samples were tested for anti-Ad5 immunoglobulin G by Virolab, Inc (Berkeley, CA) by an indirect immunofluorescence assay.[29]

Histology

Hematoxylin-and-eosin stained slides from each biopsy were prepared and evaluated for histologic grade, extent of viable tumor and necrosis, and the presence of inflammatory cell infiltrates.

Immunohistochemical staining (IHC) for p53 was performed on formalin-fixed, paraffin-embedded tissue sections by DO-1 antibody (Oncogene Science, Uniondale, NY) with an avidin-biotin-peroxidase complex method.[30] Scoring was accomplished by counting positive nuclear staining in 100 to 200 tumor cells in 10 consecutive high-power fields. Specimens were scored for the percentage of cells that expressed p53. All slides were coded, evaluated, and scored in a blinded fashion. Samples with 20% or greater of tumor cells that showed positive staining were defined as IHC-positive.

RESULTS

Treatment and Safety

A nonrandomized, phase I, dose-escalation study was conducted to ascertain the safety of Ad-p53 in resectable and nonresectable HNSCC patients. Efficacy and biodistribution were also monitored. Doses ranged from 10^6 to 10^{11} pfu, with six to 42 doses of Ad-p53 administered per patient over a course of 2 weeks to 6½ months. A total of 429 doses of Ad-p53 were administered, with a maximum total-dose per patient of 3×10^{12} pfu.

The multiple courses of direct intratumoral injections of Ad-p53 were well tolerated. Neither dose-limiting effects of Ad-p53 injection nor serious adverse events related to the study treatment occurred. Injection site pain was the most common adverse event and occurred in 19 patients, but it did not correlate with dose or anatomic site of injection. Other Ad-p53–related adverse events that occurred three or more times were seen primarily at doses of 10^{10} pfu or greater (14 of 17 events) and consisted of transient fever, headache, pain, and edema. Injection site pain and headache resolved within 24 hours, fever within 48 hours, and edema within 4 days. There was mild erythema at the site of injection with doses of 3×10^9 pfu or greater, which did not alter treatment schedules. No evidence of systemic hypersensitivity or allergic reaction was seen despite patients who received as many as seven monthly courses at 10^9 pfu, six courses at 10^{10} pfu, or four courses at 10^{11} pfu. Peri- and postoperative administration of Ad-p53 had no adverse effect on wound healing (data not shown).

Because health care providers were potentially exposed to Ad-p53 during injections, the two providers with the greatest risk of exposure were tested. Serum collected after more than 75% of the doses had been administered showed low levels of anti-Ad5 antibody and a lack of infectious Ad-p53 or Ad-p53 DNA. The level of antibody determined in sera from health care providers was within the range of all pretreatment serum values determined for all patients in the study. Urine also contained no infectious Ad-p53 or Ad-p53 DNA. These findings suggest no significant exposure of health care providers to Ad-p53 during this study.

Patient p53 Mutational Status

Patient p53 mutational status was determined both by sequencing of genomic DNA (exons 5 through 10) and by IHC. Generally, wild-type p53 protein 15 was present at very low levels that were undetectable by IHC, whereas mutant p53 protein 15 was present at much higher levels and can be detected by IHC.[31] Overall, 58% of patients were p53 mutant as determined by IHC and 48% were p53 mutant as determined by sequencing (Table 1). Agreement was observed between IHC and sequencing in 71% of the patients who could be evaluated. Patient 14 showed a mutation that led to a truncated p53 protein that could not be detected by IHC.

Clinical Results

Ad-p53 administration resulted in objective tumor regressions (Table 1) in some refractory- or recurrent-disease patients despite large tumor burdens. Of the 17 nonresectable-disease patients, two showed a greater than 50% reduction in the indicator lesion by CT scan, six showed stable disease, and nine progressed. The two patients who showed the greatest activity received doses of 10^{10} and 10^{11} pfu (Figs 2 and 3). The duration of clinical activity was 7 weeks in one patient and was documented for 18 days in the second patient, at which point the patient withdrew from the study and was lost to follow-up. The duration of stable disease in six patients was 1 to 3.5 months. Several nonresectable-disease patients showed tumor progression in nonindicator lesions, which caused morbidity and removal from the study.

Resectable-disease patients were not considered assessable for antitumor activity because of removal of the indicator lesion. However, patient 5 (treated with six doses of 10^7 pfu) showed a complete pathologic response in that no viable tumor was found in the completely resected specimen. At the time of pathologic evaluation, the tumor mass had been replaced by liquifactive necrosis. This patient remains disease free at 26 months from study entry. Resected-disease patient 10 also showed no evidence of disease at 24 months from study entry. At the time of this publication, six of the 15 resected-disease patients have died, which included one non–cancer-related death.

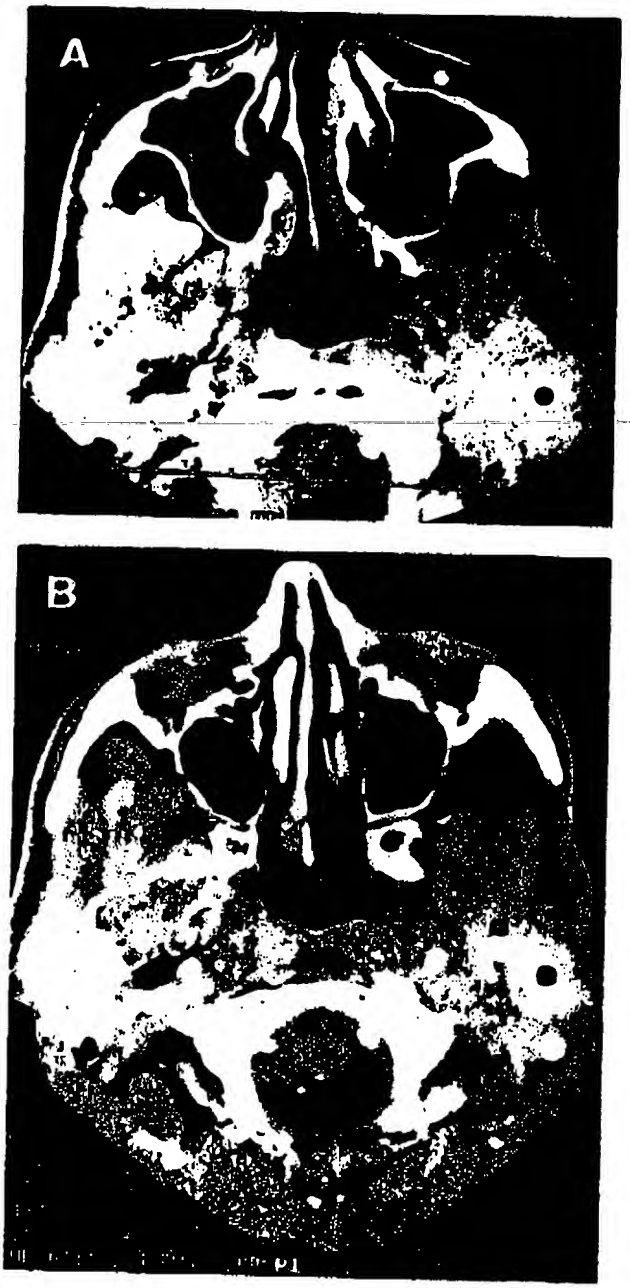

Fig 2. Axial post-contrast CT images (before and after 6 doses of 10^{10}) in patient 23, with recurrent HNSCC and previous hemimandibulectomy and flap reconstruction. (A) Pretreatment, postoperative distortion and enhancing tumor in the left condylar fossa/subtemporal region (dot). (B) Posttreatment, > 50% tumor reduction (dot).

The median survival time for all treated patients was 267 days and for all resectable-disease patients was 408 days. Median survival time for the nonresectable-disease patients was 127 days, which was consistent with other reported phase I and II studies of advanced recurrent head and neck cancer.[1,4,32] Of the 18 nonresectable-disease patients, 15 have died. However, death does not necessarily reflect progression of the lesions injected with Ad-p53 because progression also occurred at untreated sites.

Ad-p53 Transduction

Expression of the p53 transgene (Table 2) was detected by RT-PCR from patients 10 and 13 from biopsy samples taken at 4 and 48 hours after treatment, respectively (Fig 4). In contrast, biopsies from patients 5 and 8 at 1 hour after Ad-p53 delivery were negative. Tissues collected from non–Ad-p53-injected sites were used as controls. The positive biopsy sample from patient 13 was taken 67 days after the start of Ad-p53 injections (and 48 hours after the last injection) and showed transgene expression long after the development of a strong antibody response to Ad5. No transgene mRNA was detected in pretreatment biopsy samples or in non–Ad-p53-injected tumor samples (data not shown).

p53 IHC analysis was complicated by the large proportion of patients (52%) with high-positive (> 50% of cells) pretreatment values for p53. In a subset of 12 patients with pretreatment p53 values of 50% or less, three patients showed increased p53 protein expression after treatment. Figure 5 shows an increase in p53 IHC staining 48 hours after the last Ad-p53 injections compared with the prestudy biopsy sample.

Immune Response

All patients injected with doses greater than 10^7 pfu showed an increase in antiadenovirus type 5 antibody. All increases were manifested by 4 weeks, although a more limited sampling suggested the increase occurred by 1 week. The largest increase in titer was 2,048-fold greater than the prestudy levels. Antibody induction level did not correlate with Ad-p53 dose or course of treatment (data not shown).

Biodistribution

Ad-p53 DNA was detected in blood in a dose-dependent manner, as assayed by PCR (Table 3). Using this method, Ad-p53 DNA was present in blood by 30 minutes after Ad-p53 injections and was absent by 48 hours (data not shown). CPE data from four patients treated at 3×10^{10} and 10^{11} pfu showed viable Ad-p53 present at the highest levels 30 minutes after injections, a decrease of two to four orders of magnitude by 90 minutes, a further decrease to a very low or undetectable titer by 24 hours, and an absence by 48 hours after injections.

Infectious Ad-p53 was detected in urine from some patients who received doses of 3×10^9 pfu or greater and was present in urine from all patients who received doses of

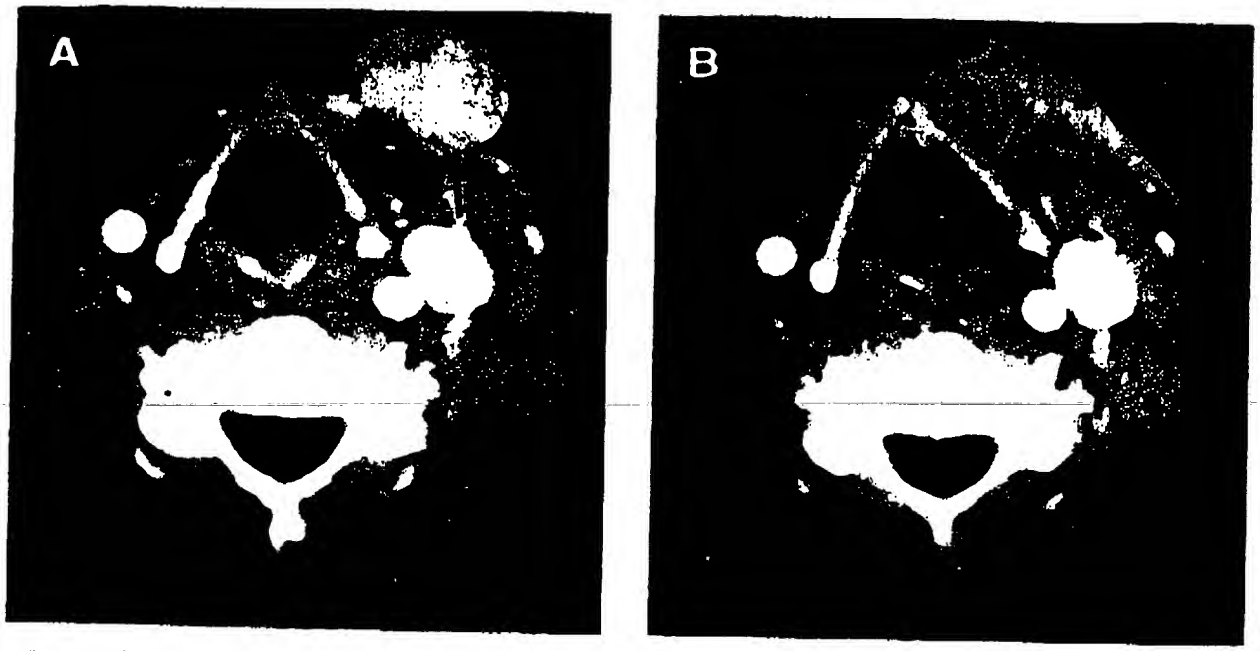

Fig 3. Axial post-contrast CT images (before and after 6 doses of 10^{11} pfu) in patient 30, with recurrent squamous cell carcinoma and previous bilateral neck dissections. (A) Pretreatment, left-sided metastatic dermal implant (arrow). (B) Posttreatment, > 50% tumor reduction (arrow).

3×10^{10} pfu or greater (Table 3). Ad-p53 was generally detected within 1 day of the beginning of Ad-p53 injections and was present throughout cycles. A representative example of the time course of Ad-p53 in urine during treatment is shown in Fig 6. The highest titer detected in urine was 10^6 pfu per 0.5 mL. Urine was free of Ad-p53 within 3 to 17 days of the last Ad-p53 injection.

Ad-p53 was also detected in sputum and/or saliva samples of the six high-dose patients tested (patients 28 through 34, who all received 10^{11} pfu). The highest titer found was 10^6 pfu per 0.5 mL. As with urine samples, Ad-p53 was generally detected within 1 day of the first injection of Ad-p53 and was present throughout each cycle. Ad-p53 was usually present for several days after the last injection of Ad-p53 and was cleared to background levels within 7 days. The time-course profiles were similar to those found for urine.

Table 2. Assay of Ad-p53 Transgene Expression by RT-PCR

Patient No.	Dose (pfu)	Time From Last Dose (hours)	Time From First Dose (days)	Anti-Ad5 Antibody*	RT-PCR
5	1×10^7	1	18	2	-
8	1×10^7	1	29	64	-
10	1×10^8	4	14	8	++
13	1×10^9	48	67	128	+

*Fold increase over pretreatment levels; ≥ four-fold represents a significant increase.

DISCUSSION

After Ad-p53 administration to patients with HNSCC, the primary consideration in this phase I clinical study was patient safety. Adverse events reported in the study were typical of this patient population. No untoward incidents or trends were noted, and no serious adverse events related to injection of Ad-p53 were detected. Thus, up to 3×10^{12} pfu of Ad-p53 could be administered without serious side effects. This level is believed to be the highest dose of adenoviral vector to be delivered to patients to date. A maximum-tolerated dose was not defined in this trial because the maximum dose of 1×10^{11} pfu led to no clinically significant side effects.

Pain at injection site was the most common treatment-related adverse event, and is believed to be related to the injection of ice-cold Ad-p53 solution. Recent stability studies indicate that chilling Ad-p53 on ice until immediately before injection is not necessary. An increase in the temperature of the injected Ad-p53 could minimize this pain in future studies.

Ad-p53 administration led to objective antitumor activity in some patients. Of the 17 nonresectable-disease patients, two showed a greater than 50% reduction of the indicator lesion, and six showed stable disease at the indicator lesion. Although resectable-disease patients could not be formally characterized for response, patient 5 had a complete pathologic response and remains disease free at 26 months.

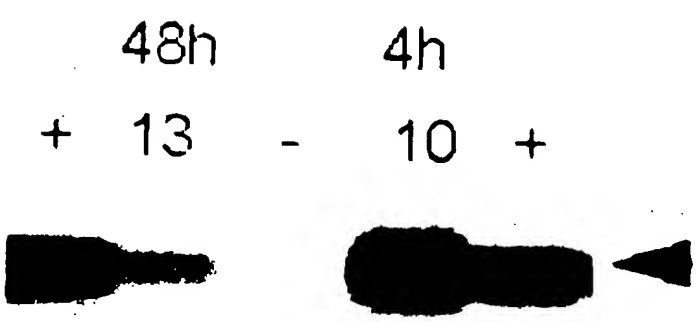

Fig 4. Demonstration of Ad-p53 transgene expression by RT-PCR and Southern blot hybridization. Patient 13 was biopsied 48 hours after treatment, and patient 10 was biopsied 4 hours after treatment. (Arrowhead), the expected size of the PCR product; (+) positive control; (−) negative control.

Patient 10 remains alive with no evidence of disease at 24 months. Only six deaths (one non–cancer related) have occurred among the 15 patients with resected disease. These responses are even more impressive given the large tumor burdens carried by most of the patients (example in Fig 1). In the nonresectable-disease patients, the Kaplan-Meier median survival time was 127 days, consistent with other phase I and phase II studies of advanced recurrent head and neck cancer.[1,4] The median survival for resectable-disease patients was 408 days, or 13.6 months, and the overall median survival was 267 days, which is about 60% longer than that reported in chemotherapy trials with a similar patient profile.[32] Although preliminary and uncontrolled, it is believed that these are encouraging data that could support evaluation of Ad-p53 in the treatment of HNSCC. These results are also in agreement with a previous study that showed that restoration of wild-type p53 expression by a retroviral vector in non–small-cell lung cancer could mediate tumor regression.[33] The design of the study does not allow us to conclude that the observed clinical activity was specifically a result of vector and transgene induction.

Patients with indicator lesion regression of 50% or more were discordant for p53 status as assayed by DNA sequencing and IHC analysis (Table 1). However, the tumors of the three patients with stable disease were wild-type for p53 as shown by both assays. In addition, patient 5, who underwent a complete histologic response and is alive with no evidence of disease at 26 months after the start of treatment, is p53 wild-type by both sequencing and IHC. These data strongly suggest that Ad-p53 can exert a significant antitumor effect on HNSCC tumors regardless of the p53 status of the tumors.

The simplest explanation for the observed responses to Ad-p53 is that p53 expression kills tumor cells. However, given previous studies on the spread of adenovirus after intratumoral injection[34] and the large sizes of the tumors in this study, one might expect a minority of tumor cells to be infected by Ad-p53. In considering other effects of Ad-p53 on the tumor, one possibility is that p53 expression may be exerting an antiangiogenic effect. Evidence that wild-type p53 decreases the expression of angiogenic factors and increases the expression of antiangiogenic factors has been presented.[35-37] Another possibility is that the adenoviral vector triggers an immune response that might adversely

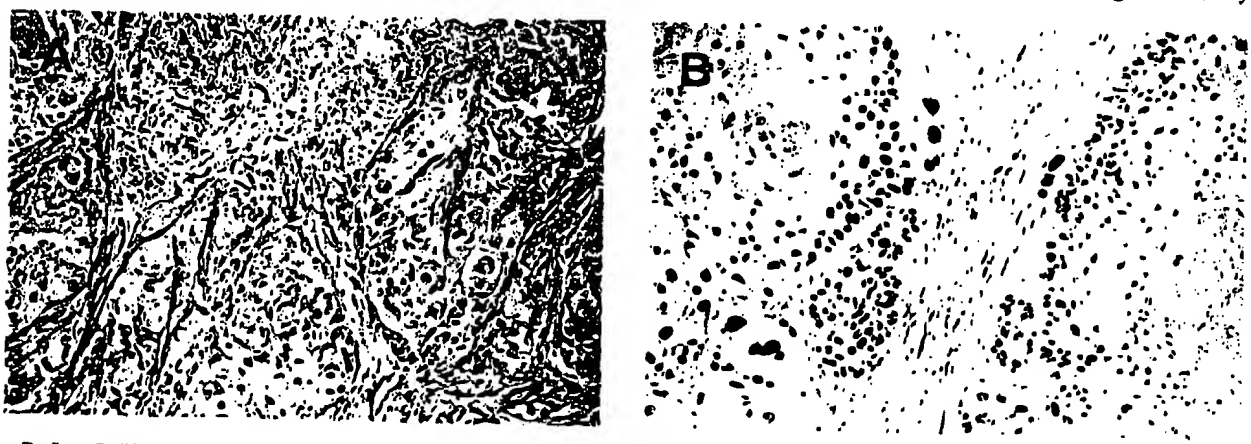

Fig 5. p53 IHC of tumor biopsy sections from patient 8 (p53 mutant, IHC positive) after 7 doses of 10^7 pfu. (A) Pretreatment biopsy. (B) Biopsy of the wound bed 48 hours after wound-bed treatment. The light to dark brown nuclear stain represents positive p53 IHC.

Table 3. Presence of Ad-p53 DNA in Body Fluids

Dose	Blood		Urine
	30-90 Minutes*	24 Hours*	
10^6	0	0	0
10^7	25	0	0
10^8	0	0	0
10^9	50	0	0
3×10^9	66	0	66
10^{10}	100	0	66
3×10^{10}	100	0	100
10^{11}	100	0	100

NOTE. Table indicates the detection of Ad-p53 DNA by PCR in serum (patients 4, 5, 7-28) and urine (all patients).
*Value indicates percentage of patients with at least one positive sample.

affect the tumor. In addition, E1-deleted, replication-defective adenoviruses can in fact replicate to some extent if the multiplicity of infection is high enough.[38] Because Ad-p53 was injected directly into tumors, the possibility of a high multiplicity of infection cannot be excluded. Local Ad-p53 replication could have several effects, which include direct killing of cells and enhancement of the host immune response.

RT-PCR showed the ability of Ad-p53 to infect tumor cells and induce expression of p53 mRNA (Fig 4). Transgene mRNA was detected in samples collected 4 and 48 hours after the last injection, but not in two samples collected 1 hour after treatment. This lack of signal in the 1-hour samples could be because of insufficient time for expression, the lower dose administered to patients in the 1-hour samples, or sample variation (see below). Interestingly, the transgene mRNA expression detected 48 hours after the last injection occurred 67 days from the start of treatment. By this time, a strong antibody response to adenovirus had occurred (Table 2), which led to the conclusion that a strong humoral immune response does not prevent transgene expression from intratumoral injection of Ad-p53.

One example of IHC that showed increased p53 expression after treatment is shown in Fig 5. Biopsies from three patients with low pretreatment p53 IHC showed increased p53 IHC after Ad-p53 injection. Several explanations are possible for the sporadic nature of the detection of increased p53 IHC after Ad-p53 injection. First, Ad-p53–infected tumor cells that undergo apoptosis are no longer present and cannot be evaluated for IHC signal. Biopsies for IHC were performed 3 days after the last injection, but recent data (not shown) suggest that earlier sampling may have detected p53 expression before cell death. Second, necrotic areas that appeared in some tumors after treatment were avoided during biopsy and histopathologic analysis. If necrotic areas were caused by Ad-p53, the biopsy procedure would be biased so as to underrepresent p53 IHC. Third, the large nature of the tumors made sampling of tumors in close proximity to sites of Ad-p53 injection difficult.

The p53 mutation spectrum in the population of treated patients was found to be consistent with earlier studies.[39] Most tumor samples (71%) analyzed for p53 status showed agreement between detection of mutations by partial genomic sequencing and IHC. This degree of concordance was also consistent with other reports.[40] The subset of patient biopsy specimens for which IHC and DNA sequencing disagreed may be because of the inability of sequencing technologies to detect a low percentage of tumor DNA in biopsy samples, mutations outside of exons 5 through 10,[39] accumulation of wild-type p53 because of MDM2 overexpression,[41] and p53 mutant proteins that do not show increased stability.[42] As evidence of the first possibility, when a tumor sample from patient 23 was screened with a more sensitive sequencing method (OncorMed, Gaithersburg, MD), a mutation was indeed detected in exon 8.

The data presented from this study indicate much more extensive biodistribution and dissemination than had previously been detected. The appearance of Ad-p53 in blood was relatively constant and appeared in almost every patient 30 to 90 minutes after injection at doses greater than 10^9 pfu. Infectious vector had not previously been detected in patient blood after the administration of up to 2×10^9 pfu to nasal and/or bronchial epithelia,[43,44] a dose level that caused detectable vector in blood in this study (Table 3).

Wild-type Ad5 had been reported to be present in urine in rare cases of acute infections in severely immunocompromised patients.[45] However, the substantial levels and duration of excretion of the replication-defective Ad-p53 in urine were remarkable. The consistent detection of Ad-p53 in urine at doses of 3×10^9 pfu or greater is in contrast to other studies[43,44,46] that did not detect vector in urine after administration of up to 2×10^{10} pfu. Detection of Ad-p53 in

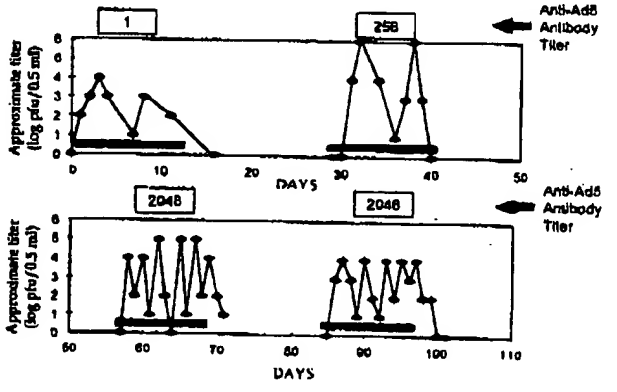

Fig 6. Presence of infectious Ad-p53 in urine from patient 34 as assayed by CPE. Day 1, first day of treatment; solid bars parallel to the X-axis mark treatment courses 1-4. Boxed numbers above the graph denote anti-Ad5 antibody titers, measured at the start of each course.

blood and urine in this study may be because of more sensitive detection methods, more facile dissemination from tumoral injection sites compared with the nasal/bronchial administration route used by others, or the low number of patients treated at high doses in other studies.

The presence of Ad-p53 in UATS samples is not surprising for patients whose tumors intrude into the oral cavity or upper airway, particularly for those patients in whom the tumor was injected through the oral cavity or airway. However, several of the tumors were not contiguous with the airway, which suggests that Ad-p53 can gain access to the airway indirectly through systemic and probably blood-borne biodistribution.

The fluctuations in urine and UATS Ad-p53 levels during courses of Ad-p53 treatment and the large variation in the amount of time necessary to return to baseline after Ad-p53 administration (1 to 17 days) may be because of heterogeneity in tumor location and size. Also, the urine and UATS samples were heterogeneous in that the urine samples were first morning voids and not full 24-hour collections and the UATS samples were either sputum, saliva, or oral cavity wash, which depended on the patient.

As expected, almost all patients developed anti-Ad5 antibodies over the course of treatment. This response did not seem to be deleterious to the patients. In addition, the patient immune response did not block transduction and expression of the p53 transgene. Numerous animal studies with replication-defective adenovirus have shown decreased transgene expression as a result of an immune response. Furthermore, these studies suggested a total lack of transduction as a result of repeated doses that were also believed to be because of an immune response.[47,48]

The patient immune response also did not prevent the appearance of Ad-p53 in blood, urine, and UATS (Fig 6, data not shown). Whereas it is known that patients may shed wild-type adenovirus for many months after an infection and in the presence of a humoral immune response,[45,49] it was originally hypothesized that dissemination would be affected at least to some effect by an immune response. However, the previous studies involved intravenous administration of adenoviral vectors in mice, which may not be relevant to this study. First, intratumoral injection may differ substantially from systemic administration. Bramson et al[50] recently showed efficient transgene expression after administration of an adenoviral vector by intratumoral injection in mice previously immunized with Ad5. Therefore, tumors may represent immunologic sanctuaries. Second, the use of mice as a model organism may be problematic, because the biologic activity of adenovirus in humans and mice is very different in that mice are not permissive for Ad5 replication.[51]

The promising results noted in this study suggest that Ad-p53 may show activity in patients with HNSCC; therefore, further study is needed and is underway. Established patient benefit is not clearly or routinely evident. Possible applications are nevertheless attractive. Brennan et al[52] used a sensitive PCR-based assay to detect tumor-specific p53 mutations in resected tumor margins from patients whose tumor margins were histopathologically free from residual tumor. They found that patients with molecular evidence of tumor cells (as detected by tumor-specific p53 mutations) have a higher likelihood of recurrence and mortality. Positive RT-PCR results that showed transduction in margins after surgery in this study (patient 10; Table 2) give direct evidence that surgical intervention may allow gene transfer into surgical sites of microscopic tumor. Given that peri- and postoperative administration of Ad-p53 had no adverse effect on wound healing, this approach may be valuable as adjuvant therapy in areas of microscopic residual disease at tumor margins to prevent recurrence and avoid further surgical ablation of normal tissue. The impact of gene transfer strategies additionally requires significant development in delivery methods that can effectively and efficiently distribute the vector to other lesions, mucosa, and potential microscopic disease sites.

Other possible future directions for Ad-p53 gene therapy include combination therapy with radiation or cytotoxic agents, as suggested by the enhanced antitumoral effects of combination treatments in preclinical models.[21-23] Also, the identification of p53 mutations in head and neck premalignancies[53] suggests that this approach may be therapeutic in severely dysplastic preinvasive lesions of this region.

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REFERENCES

1. Vokes EE, Weichselbaum RR, Lippman SM, et al: Head and neck cancer. N Engl J Med 328:184-194, 1993
2. Day GL, Blot WJ, Shore RE, et al: Second cancers following oral and pharyngeal cancers: Role of tobacco and alcohol. J Natl Cancer Inst 86:131-137, 1994
3. Parker SL, Tong T, Bolden S, et al: Cancer statistics, 1997. CA Cancer J Clin 47:5-27, 1997
4. Clayman GL, Lippman SM, Laramore GE, et al: Head and neck cancer, in Holland JF, Frei E III, Bast RC, et al (eds): Cancer Medicine (ed 4). Baltimore, MD, Williams & Wilkins, 1997, pp 1645-1710
5. Raybaud-Diogene H, Tetu B, Morency R, et al: p53 overexpression in head and neck squamous cell carcinoma: Review of the literature. Eur J Cancer B Oral Oncol 32B:143-149, 1996
6. Ko LJ, Prives C: p53: Puzzle and paradigm. Genes Dev 10:1054-1072, 1996
7. Hansen R, Oren M: p53; From inductive signal to cellular effect. Curr Opin Genet Dev 7:46-51, 1997
8. Berkner KL: Development of adenovirus vectors for the expression of heterologous genes. Biotechniques 6:616-629, 1988
9. Horwitz MS: Adenoviruses, in Fields BN, Knipe DM, Howley PM, et al (eds): Fields Virology (ed 3). Philadelphia, PA, Lippincott-Raven Publishers, 1996, pp 2149-2171
10. Chen P-L, Chen Y, Bookstein R, et al: Genetic mechanisms of tumor suppression by the human p53 gene. Science 250:1576-1580, 1990
11. Yonish-Rouach E, Resnitzky D, Rotem J, et al: Wild-type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6. Nature 352:345-347, 1991
12. Shaw P, Bovey R, Tardy S, et al: Induction of apoptosis of wild-type p53 in a human colon tumor-derived cell line. Proc Natl Acad Sci USA 89:4495-4499, 1992
13. Fujiwara T, Grimm EA, Mukhopadhyay T, et al: A retroviral wild-type p53 expression vector penetrates human lung cancer spheroids and inhibits growth by inducing apoptosis. Cancer Res 53:4129-4133, 1993
14. Clayman GL, El-Naggar AK, Roth JA, et al: In vivo molecular therapy with p53 adenovirus for microscopic residual head and neck squamous carcinoma. Cancer Res 55:1-6, 1995
15. Hamada K, Alemany R, Zhang W-W, et al: Adenovirus-mediated transfer of a wild-type p53 gene and induction of apoptosis in cervical cancer. Cancer Res 56:3047-3054, 1996
16. Liu TJ, El-Naggar AK, McDonnell TJ, et al: Apoptosis induction mediated by wild-type p53 adenoviral gene transfer in squamous cell carcinoma of the head and neck. Cancer Res 55:3117-3122, 1995
17. Noble JR, Willetts KE, Mercer WE, et al: Effects of exogenous wild-type p53 on a human lung carcinoma cell line with endogenous wild-type p53. Exp Cell Res 203:297-304, 1992
18. Liu T-J, Zhang W-W, Taylor DL, et al: Growth suppression of human head and neck cancer cells by the introduction of a wild-type p53 gene via a recombinant adenovirus. Cancer Res 54:3662-3667, 1994
19. Nguyen DM, Spitz FR, Yen N, et al: Gene therapy for lung cancer: Enhancement of tumor suppression by a combination of sequential systemic cisplatin and adenovirus-mediated p53 gene transfer. J Thorac Cardiovasc Surg 112:1372-137, 1996
20. Nielsen LL, Dell J, Maxwell E, et al: Efficacy of p53 adenovirus-mediated gene therapy against human breast cancer xenografts. Cancer Gene Ther 4:129-138, 1997
21. Fujiwara T, Grimm EA, Mukhopadhyay T, et al: Induction of chemosensitivity in human lung cancer cells in vivo by adenovirus-mediated transfer of the wild-type p53 gene. Cancer Res 54:2287-2291, 1994
22. Pirollo KF, Hao Z, Rait A, et al: p53-mediated sensitization of squamous cell carcinoma of the head and neck to radiotherapy. Oncogene 14:1735-1746, 1997
23. Spitz FR, Nguyen D, Skibber JM, et al: Adenoviral-mediated wild-type p53 gene expression sensitizes colorectal cancer cells to ionizing radiation. Clin Cancer Res 2:1665-1671, 1996
24. Zhang WW, Fang X, Branch CD, et al: Generation and identification of recombinant adenovirus by liposome-mediated transfection and PCR analysis. Biotechniques 15:868-872, 1993
25. Ajani JA, Welch SR, Raber MN, et al: Comprehensive criteria for assessing therapy-induced toxicity. Cancer Invest 8:147-159, 1990
26. Ausubel FM, Brent R, Kingston RE, et al: Current Protocols in Molecular Biology. New York, NY, John Wiley & Sons, Inc, 1995
27. Graham FL, Smiley J, Russell WC, et al: Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 36:59-72, 1997
28. Cunningham R, Harris A, Frankton A, et al: Detection of cytomegalovirus using PCR in serum from renal transplant recipients. J Clin Pathol 48:575-577, 1995
29. Lennette EH, Lennette DA, Lennette ET: Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections (ed 7). Washington, DC, American Public Health Association, 1995
30. Hsu SM, Raine L, Fanger H: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29:577-580, 1981
31. Oren M, Reich NC, Levine AJ: Regulation of the cellular p53 tumor antigen in teratocarcinoma cells and their differentiated progeny. Mol Cell Biol 2:443-449, 1982
32. Schornagel JH, Verweij J, de Mulder PHM, et al: Randomized phase III trial of edatrexate versus methotrexate in patients with metastatic and/or recurrent squamous cell carcinoma of the head and neck: A European Organization for Research and Treatment of Cancer Head and Neck Cancer Cooperative Group study. J Clin Oncol 13:1649-1655, 1995
33. Roth JA, Nguyen D, Lawrence DD, et al: Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer. Nature Med 2:985-991, 1996
34. Cusack JC, Spitz FR, Nguyen D, et al: High levels of gene transduction in human lung tumors following intralesional injection of recombinant adenovirus. Cancer Gene Ther 3:245-249, 1996
35. Van Meir EG, Polverini PJ, Chazin VR, et al: Release of an inhibitor of angiogenesis upon induction of wild-type p53 expression in glioblastoma cells. Nat Genet 8:171-176, 1994
36. Dameron KM, Volpert OV, Tainsky MA, et al: Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science 265:1582-1584, 1994
37. Mukhopadhyay D, Tsiokas L, Sukhatme VP: Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. Cancer Res 55:6161-6165, 1995
38. Shenk T, Jones N, Colby W, et al: Functional analysis of adenovirus-5 host-range deletion mutants defective for transformation of rat embryo cells. Cold Spring Harb Symp Quant Biol 44:367-375, 1980
39. Hainaut P, Soussi T, Shomer B, et al: Database of p53 gene somatic mutations in human tumors and cell lines: Updated compilation and future prospects. Nucleic Acids Res 25:151-157, 1997
40. Sjogren S, Inganas M, Norberg T, et al: The p53 gene in breast

cancer: Prognostic value of complementary DNA sequencing versus immunohistochemistry. J Natl Cancer Inst 88:173-182, 1996

41. Oliner JD, Kinzler KW, Meltzer PS, et al: Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature 358:80-83, 1992

42. Hollstein M, Sidransky D, Vogelstein B, et al: p53 mutations in human cancers. Science 253:49-53, 1991

43. Crystal R, McElvaney N, Rosenfeld M, et al: Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. Nat Genet 8:42-51, 1994

44. Zabner J, Ramsey BW, Meeker DP, et al: Repeat administration of an adenovirus vector encoding cystic fibrosis transmembrane conductance regulator to the nasal epithelium of patients with cystic fibrosis. J Clin Invest 97:1504-1511, 1996

45. Hierholzer JC: Adenoviruses in the immunocompromised host. Clin Microbiol Rev 5:262-274, 1992

46. Knowles MR, Hohneker KW, Zhou Z, et al: A controlled study of adenoviral-vector-mediated gene transfer in the nasal epithelium of patients with cystic fibrosis. New Engl J Med 333:823-831, 1995

47. Yang Y, Nunes FA, Berencsi K, et al: Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc Natl Acad Sci USA 91:4407-4411, 1994

48. Dai Y, Schwarz EM, Gu D, et al: Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: Tolerization of factor IX and vector antigens allows for long-term expression. Proc Natl Acad Sci USA 92:1401-1405, 1995

49. Fox J, Brandt C, Wassermann F, et al: The Virus Watch Program: A continuing surveillance of viral infections in metropolitan families. Am J Epidemol 89:25-50, 1969

50. Bramson JL, Hitt M, Gauldie J, et al: Pre-existing immunity to adenovirus does not prevent tumor regression following intratumoral administration of a vector expressing IL-12 but inhibits virus dissemination. Gene Ther 4:1069-1076, 1997

51. Huebner RJ, Rowe WP, Chanock RM: Newly recognized respiratory tract viruses. Annu Rev Microbiol 12:49-76, 1958

52. Brennan JA, Mao L, Hruban RH, et al: Molecular assessment of histopathologic staging. New Engl J Med 332:429-435, 1995

53. Boyle JO, Hakim J, Koch W, et al: The incidence of p53 mutation increases with progression of head and neck cancer. Cancer Res 53:4477-4480, 1993

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
GARY L. CLAYMAN

Serial No.: 08/758,033

Filed: November 27, 1996

For: METHOD AND COMPOSITION FOR THE DIAGNOSIS AND TREATMENT OF CANCER

Group Art Unit: 1632

Examiner: K. Hauda

Atty. Dkt. No.: INRP:041/HYL

CERTIFICATE OF MAILING
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class Mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on the date below:

11/9/99
DATE

SIGNATURE

AMENDMENT UNDER 37 C.F.R. §1.116

BOX AF
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

This is in amendment is in partial response to the final Office Action mailed on April 12, 1999, regarding the above-captioned application. The amendment is filed concurrent with appellant's brief, which is due on November 8, 1999, by virtue of the enclosed petition for extension of time and payment of requisite fees. Please amend the application as follows.

A: 165742()
A: 229887(4XDR011.DOC)

AMENDMENTS

In the Claims:

Please cancel claims 33-35, 69-72, 104-107, 133-136, 140, 141, 144 and 145 and amend the remaining claims as indicated below:

1. (Three times amended) A method of inhibiting growth of a p53-positive tumor cell in a [mammlian] mammalian subject with a solid tumor comprising the steps of :

(a) providing a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter; and

(b) directly administering said viral expression construct to said tumor *in vivo*, the administration resulting in expression of said functional p53 polypeptide in cells of said tumor and inhibition of tumor cell growth,

wherein said tumor comprises cells that express a functional p53 polypeptide.

14. (Amended) The method of claim 11, wherein the amount of adenovirus [administered] in each [contacting] administration is between about 10^7 and 10^{12} pfu.

64. (Amended) The method of claim 61, wherein said DNA damaging agent is contacted [contacting] before and after resection.

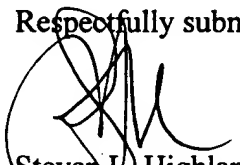
109. (Twice amended) A method of inhibiting tumor cell growth in a mammalian subject having a solid tumor comprising the step of continuously perfusing a tumor site in said patient with a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter, the administration resulting in expression of said functional p53 polypeptide in cells of said tumor and inhibition of their growth.

REMARKS

Appellant is resubmitting the amendments filed with their previous response. The only alteration is the elimination of an amendment to claim 109 inserting the term "catheter." The examiner refused entry of the amendment on the ground that introduction of the term "catheter" would require a new search. Thus, it is believed that the revised amendment should be acceptable as it merely cancels claims and makes minor wording changes to several other claims. No new matter is added by the amendment, no new search is required, and no new issues are raised thereby.

Should Examiner Hauda have any questions regarding this amendment, she is invited to contact the undersigned at the telephone number listed below.

Respectfully submitted,



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A: 165742Q
A: 229887(4XDR011.DOC)

SUMMARY OF PENDING CLAIMS

1. (Three times amended) A method of inhibiting growth of a p53-positive tumor cell in a [mammlian] mammalian subject with a solid tumor comprising the steps of:

   (a) providing a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter; and

   (b) directly administering said viral expression construct to said tumor *in vivo*, the administration resulting in expression of said functional p53 polypeptide in cells of said tumor and inhibition of tumor cell growth,

   wherein said tumor comprises cells that express a functional p53 polypeptide.

2. The method of claim 1, wherein said tumor is selected from the group consisting of a carcinoma, a glioma, a sarcoma, and a melanoma.

3. The method of claim 1, wherein said tumor cell is malignant.

4. The method of claim 1, wherein said tumor cell is benign.

5. The method of claim 1, wherein said tumor is a tumor of the lung, skin, prostate, liver, testes, bone, brain, colon, pancreas, head and neck, stomach, ovary, breast or bladder.

6. The method of claim 1, wherein said viral expression construct is selected from the group consisting of a retroviral vector, an adenoviral vector and an adeno-associated viral vector.

7. The method of claim 6, wherein said viral vector is a replication-deficient adenoviral vector.

8. The method of claim 7, wherein said replication-deficient adenoviral vector is lacking at least a portion of the E1-region.

9. The method of claim 8, wherein said promoter is a CMV IE promoter.

10. The method of claim 1, wherein said subject is a human.

11. The method of claim 7, wherein the expression vector is administered to said tumor at least a second time.

12. The method of claim 11, wherein said tumor is resected following at least a second administration, and an additional administration is effected subsequent to said resection.

13. The method of claim 1, wherein said expression vector is administered in a volume of about 3 ml. to about 10 ml.

14. (Amended) The method of claim 11, wherein the amount of adenovirus [administered] in each [contacting] administration is between about 10^7 and 10^{12} pfu.

16. The method of claim 1, wherein the expression construct is injected into a natural or artificial body cavity.

17. The method of claim 16, wherein said injection comprises continuous perfusion of said natural or artificial body cavity.

18. The method of claim 16, wherein said contacting is via injection into an artificial body cavity resulting from tumor excision.

19. The method of claim 1, wherein the *p53*-encoding polynucleotide is tagged so that expression of *p53* from said expression vector can be detected.

20. The method of claim 19, wherein the tag is a continuous epitope.

26. The method of claim 1, wherein said tumor is contacted with said expression construct at least twice.

27. The method of claim 26, wherein said multiple injections comprise about 0.1-0.5 ml volumes spaced about 1 cm apart.

28. The method of claim 1, further comprising contacting said tumor with a DNA damaging agent.

29. The method of claim 28, wherein said DNA damaging agent is a radiotherapeutic agent.

30. The method of claim 29, wherein said radiotherapeutic agent is selected from the group consisting of γ-irradiation, x-irradiation, uv-irradiation and microwaves.

31. The method of claim 28, wherein said DNA damaging agent is a chemotherapeutic agent.

32. The method of claim 31, wherein said chemotherapeutic agent is selected from the group consisting of adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, verapamil, doxorubicin, podophyllotoxin and cisplatin.

33. (Canceled) The method of claim 1, further comprising contacting said tumor with a cytokine.

34. (Canceled) The method of claim 1, further comprising contacting said tumor with a second therapeutic gene other than a gene encoding a *p53* polypeptide.

35. (Canceled) The method of claim 34, wherein said second therapeutic gene is selected from the group consisting of a Dp gene, p21, p16, p27, E_2F, Rb, APC, DC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, *ras*, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl*, *abl*, Bax, Bcl-X_s and E1A.

36. The method of claim 1, wherein said tumor is located into a body cavity selected from the group consisting of the mouth, pharynx, esophagus, larynx, trachea, pleural cavity, peritoneal cavity, bladder interior and colon lumen.

37. The method of claim 11, wherein said tumor is contacted with said expression construct at least six times within a two week treatment regimen.

38. A method for inhibiting microscopic residual tumor cell growth in a mammalian subject comprising the steps of:

(a) identifying a mammalian subject having a resectable tumor:

(b) resecting said tumor; and

(c) administering to a tumor bed revealed by resection a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter, the administration resulting in expression of said functional p53 polypeptide in said tumor cells and inhibition of their growth.

39. The method of claim 38, wherein said resectable tumor is a squamous cell carcinoma.

40. The method of claim 38, wherein the endogenous *p53* of said resectable tumor is mutated.

41. The method of claim 38, wherein the endogenous *p53* of said resectable tumor is wild-type.

42. The method of claim 38, wherein said tumor is a tumor of the lung, skin, prostate, liver, testes, bone, brain, colon, pancreas, head and neck, stomach, ovary, breast or bladder.

43. The method of claim 38, wherein said viral expression construct is selected from the group consisting of a retroviral vector, an adenoviral vector and an adeno-associated viral vector.

44. The method of claim 43, wherein said adenoviral vector is a replication-deficient adenoviral vector.

45. The method of claim 44, wherein said replication-deficient adenoviral vector is lacking at least a portion of the E1-region.

46. The method of claim 38, wherein said promoter is a CMV IE promoter.

47. The method of claim 38, wherein the resulting tumor bed is contacted with said expression construct at least twice.

48. The method of claim 38, wherein said expression construct is contacted with said tumor bed prior to closing of the incision.

49. The method of claim 44, wherein said the tumor bed is contacted with from about 10^6 to about 10^9 infectious adenoviral particles.

50. The method of claim 47, further comprising contacting said tumor with said expression construct prior to resecting said tumor.

51. The method of claim 50, wherein said tumor is injected with said expression construct.

52. The method of claim 51, wherein said tumor is injected with about 10^6 to about 10^9 infectious adenoviral particles.

53. The method of claim 51, wherein said tumor is injected with a total of about 1 ml to about 10 ml.

54. The method of claim 51, wherein said tumor is injected at least twice.

55. The method of claim 54, wherein each of said injections comprise about 0.1 ml to about 0.5 ml volumes spaced about 1 cm apart.

56. The method of claim 38, wherein the resulting tumor bed is contacted with said expression construct through a catheter.

57. The method of claim 54, wherein said contacting comprises about 10^6 to about 10^9 infectious adenoviral particles.

58. The method of claim 54, wherein said expression construct is contacted with said tumor in total of about 3 ml to about 10 ml.

59. The method of claim 38, wherein the *p53* polynucleotide is tagged so that expression of a *p53* polypeptide can be detected.

60. The method of claim 59, wherein the tag is a continuous epitope.

61. The method of claim 38, further comprising contacting said tumor with a DNA damaging agent.

62. The method of claim 61, wherein said DNA damaging agent is contacted before resection.

63. The method of claim 61, wherein said DNA damaging agent is contacted after resection.

64. (Amended) The method of claim 61, wherein said DNA damaging agent is contacted [contacting] before and after resection.

65. The method of claim 61, wherein said DNA damaging agent is a radiotherapeutic agent.

66. The method of claim 65, wherein said radiotherapeutic agent is selected from the group consisting of γ-irradiation, x-irradiation, uv-irradiation and microwaves.

67. The method of claim 61, wherein said DNA damaging agent is a chemotherapeutic agent.

68. The method of claim 67, wherein said chemotherapeutic agent is selected from the group consisting of adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, verapamil, doxorubicin, podophyllotoxin and cisplatin.

69. (Canceled) The method of claim 38, further comprising contacting said tumor with a cytokine.

70. (Canceled) The method of claim 69, wherein said cytokine is selected from the group consisting of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, TGF-β, GM-CSF, M-CSF, TNFα, TNFβ, LAF, TCGF, BCGF, TRF, BAF, BDG, MP, LIF, OSM, TMF, PDGF, IFN-α, IFN-β and IFN-γ.

71. (Canceled) The method of claim 38, further comprising contacting said tumor with a second therapeutic gene other than a gene encoding a *p53* polypeptide.

72. (Canceled) The method of claim 71, wherein said second therapeutic gene is selected from the group consisting of a Dp gene, p21, p16, p27, E_2F, Rb, APC, DC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, *ras*, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl*, *abl*, Bax, Bcl-X_s and E1A.

73. The method of claim 38, wherein said tumor is located into a body cavity selected from the group consisting of the mouth, pharynx, esophagus, larynx, trachea, pleural cavity, peritoneal cavity, bladder interior and colon lumen.

74. A method for inhibiting growth of a p53-positive tumor cell in a mammalian subject having a solid tumor comprising the steps of:

(a) surgically revealing said tumor; and

(b) directly administering to said tumor a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter, the administration resulting in

expression of said functional p53 polypeptide in said tumor cells and inhibition of their growth.

75. The method of claim 74, wherein said tumor is malignant.

76. The method of claim 74, wherein said tumor is a squamous cell carcinoma.

77. The method of claim 74, wherein said tumor is benign.

80. The method of claim 74, wherein said tumor is a tumor of the lung, skin, prostate, liver, testes, bone, brain, colon, pancreas, head and neck, stomach, ovary, breast or bladder.

81. The method of claim 74, wherein said viral expression construct is selected from the group consisting of a retroviral vector, an adenoviral vector and an adeno-associated viral vector.

82. The method of claim 81, wherein said adenoviral vector is a replication-deficient adenoviral vector.

83. The method of claim 82, wherein said replication-deficient adenoviral vector is lacking at least a portion of the E1-region.

84. The method of claim 74, wherein said promoter is a CMV IE promoter.

85. The method of claim 74, wherein said tumor is contacted with said expression construct at least twice.

86. The method of claim 74, wherein said expression construct is contacted with said tumor prior to close of the incision.

87. The method of claim 82, wherein said tumor is contacted with from about 10^6 to about 10^9 infectious adenoviral particles.

88. The method of claim 74, wherein said tumor is contacted with said expression construct in a total of about 1 ml to about 10 ml.

89. The method of claim 74, wherein said tumor is injected at least twice.

90. The method of claim 89, wherein each of said injections comprise about 0.1 ml to about 0.5 ml volumes spaced about 1 cm apart.

91. The method of claim 74, wherein said tumor is contacted with said expression construct through a catheter.

92. The method of claim 91, wherein said tumor is contacted with about 10^6 to about 10^9 infectious adenoviral particles.

93. The method of claim 91, wherein said tumor is contacted with an expression construct in a total of about 3 ml to about 10 ml.

94. The method of claim 74, wherein the *p53* polynucleotide is tagged so that expression of a *p53* polypeptide can be detected.

95. The method of claim 94, wherein the tag is a continuous epitope.

96. The method of claim 74, further comprising contacting said tumor with a DNA damaging agent.

97. The method of claim 96, wherein said DNA damaging agent is contacted with said tumor before resection.

98. The method of claim 96, wherein said DNA damaging agent is contacted with said tumor after resection.

99. The method of claim 96, wherein DNA damaging agent is contacted with said tumor before and after resection.

100. The method of claim 96, wherein said DNA damaging agent is a radiotherapeutic agent.

101. The method of claim 100, wherein said radiotherapeutic agent is selected from the group consisting of γ-irradiation, x-irradiation, uv-irradiation and microwaves.

102. The method of claim 96, wherein said DNA damaging agent is a chemotherapeutic agent.

103. The method of claim 102, wherein said chemotherapeutic agent is selected from the group consisting of adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, verapamil, doxorubicin, podophyllotoxin and cisplatin.

104. (Canceled) The method of claim 74, further comprising contacting said tumor with a cytokine.

105. (Canceled) The method of claim 104, wherein said cytokine is selected from the group consisting of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, TGF-β, GM-CSF, M-CSF, TNFα, TNFβ, LAF, TCGF, BCGF, TRF, BAF, BDG, MP, LIF, OSM, TMF, PDGF, IFN-α, IFN-β, and IFN-γ.

106. (Canceled) The method of claim 74, further comprising contacting said tumor with a second therapeutic gene other than a gene encoding a *p53* polypeptide.

107. (Canceled) The method of claim 106, wherein said second therapeutic gene is selected from the group consisting of a Dp gene, p21, p16, p27, E_2F, Rb, APC, DC, NF-1, NF-2, WT-1,

MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, *ras*, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl*, *abl*, Bax, Bcl-X_s and E1A.

108. The method of claim 74, wherein said tumor is located in a body cavity selected from the group consisting of the mouth, pharynx, esophagus, larynx, trachea, pleural cavity, peritoneal cavity, bladder interior and colon lumen.

109. (Twice amended) A method of inhibiting tumor cell growth in a mammalian subject having a solid tumor comprising the step of continuously perfusing a tumor site in said patient with a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter, the administration resulting in expression of said functional p53 polypeptide in cells of said tumor and inhibition of their growth.

110. The method of claim 109, wherein said tumor is malignant.

111. The method of claim 109, wherein said tumor is a squamous cell carcinoma.

112. The method of claim 109, wherein said tumor is benign.

113. The method of claim 109, wherein the endogenous *p53* of said tumor is mutated.

114. The method of claim 109, wherein the endogenous *p53* of said tumor is wild-type.

115. The method of claim 109, wherein said tumor is a tumor of the lung, skin, prostate, liver, testes, bone, brain, colon, pancreas, head and neck, stomach, ovary, breast or bladder.

116. The method of claim 116, wherein said viral expression construct is selected from the group consisting of a retroviral vector, an adenoviral vector and an adeno-associated viral vector.

117. The method of claim 116, wherein said adenoviral vector is a replication-deficient adenoviral vector.

118. The method of claim 117, wherein said replication-deficient adenoviral vector is lacking at least a portion of the E1-region.

119. The method of claim 109, wherein said promoter is a CMV IE promoter.

120. The method of claim 109, wherein said tumor site is perfused from about one to two hours.

121. The method of claim 109, wherein said subject is a human.

122. The method of claim 109, wherein said tumor site is contacted with said expression vector through a catheter.

123. The method of claim 109, wherein the *p53* polynucleotide is tagged so that expression of a *p53* polypeptide can be detected.

124. The method of claim 123, wherein the tag is a continuous epitope.

125. The method of claim 109, further comprising contacting said tumor with a DNA damaging agent.

126. The method of claim 125, wherein said tumor site is contacted with said DNA damaging agent before resection.

127. The method of claim 125, wherein said tumor site is contacted with said DNA damaging agent after resection.

128. The method of claim 125, wherein said tumor site is contacted with said DNA damaging agent before and after resection.

129. The method of claim 125, wherein said DNA damaging agent is a radiotherapeutic agent.

130. The method of claim 129, wherein said radiotherapeutic agent is selected from the group consisting of γ-irradiation, x-irradiation, uv-irradiation and microwaves.

131. The method of claim 125, wherein said DNA damaging agent is a chemotherapeutic agent.

132. The method of claim 131, wherein said chemotherapeutic agent is selected from the group consisting of adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, verapamil, doxorubicin, podophyllotoxin and cisplatin.

133. (Canceled) The method of claim 109, further comprising contacting said tumor with a cytokine.

134. (Canceled) The method of claim 133, wherein said cytokine is selected from the group consisting of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, TGF-β, GM-CSF, M-CSF, TNFα, TNFβ, LAF, TCGF, BCGF, TRF, BAF, BDG, MP, LIF, OSM, TMF, PDGF, IFN-α, IFN-β, and IFN-γ.

135. (Canceled) The method of claim 74, further comprising contacting said tumor with a second therapeutic gene other than a gene encoding a *p53* polypeptide.

136. (Canceled) The method of claim 135, wherein said second therapeutic gene is selected from the group consisting of a Dp gene, p21, p16, p27, E_2F, Rb, APC, DC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, *ras*, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl*, *abl*, Bax, Bcl-X_s and E1A.

137. The method of claim 109, wherein said tumor is located into a body cavity selected from the group consisting of the mouth, pharynx, esophagus, larynx, trachea, pleural cavity, peritoneal cavity, bladder interior and colon lumen.

138. The method of claim 1, wherein said expression vector is administered topically.

139. The method of claim 1, wherein said expression vector is administered intratumorally.

140. (Canceled) The method of claim 1, wherein said expression vector is administered intravenously.

141. (Canceled) The method of claim 1, wherein said expression vector is administered orally.

142. The method of claim 74, wherein said expression vector is administered topically.

143. The method of claim 74, wherein said expression vector is administered intratumorally.

144. (Canceled) The method of claim 74, wherein said expression vector is administered intravenously.

145. (Canceled) The method of claim 74, wherein said expression vector is administered orally.